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NATIONAL CANCER INSTITUTE  
ANNUAL REPORT

October 1, 1986 through September 30, 1987

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM-07183-01 PRB

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The influence of molecular structure on chemical and biological properties

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI : Frank R. Quinn Chemist PRB,NCI

## COOPERATING UNITS (if any)

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Pharmaceutical Resources Branch

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## INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	

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 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

X-ray crystallographic, energy minimization and quantum mechanical calculations been employed on compounds of biological interest to give insights into and explanation of their modes of behavior.

Various compounds showing promise against the AIDS virus are being systematically investigated to obtain structural and electronic properties which may help elucidate the mechanism of their action and thus lead to improved analogs. The x-ray structures of 2',3'-dideoxyadenosine (DDA) and 2',3'-dideoxycytidine (DDC) have been determined. Strain energies and quantum calculations have been carried out on these compounds. Calculations have been completed on the eight possible epimers of 3'-azido-2',3'-dideoxythymidine (AZT), as well as the corresponding bases: cytidine, adenosine, inosine, 2'-deoxycytidine and 3'-amino-3'-deoxyadenosine for comparison.

Strain energies and quantum calculations on colchicine, which binds to tubulin, and isocolchicine, which does not, have been completed. Isocolchicine is more strained by about 8 kcals./mol. The factor which differentiates the binding ability of the two isomers seems to be different inter-oxygen distances which affect hydrogen bonding ability in isocolchicine.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CM 06162-03 LBC

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October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacology of Antitumor Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R.L. Cysyk Chief LBC, NCI

Others: J.D. Moyer Staff Fellow LBC, NCI  
J.D. Strong Sr. Investigator LBC, NCI  
C.A. Chisena Biologist LBC, NCI

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Laboratory of Biological Chemistry

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 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cyclopentenyl cytosine, one of a new series of nucleoside analogues, was found to have marked in vivo antitumor activity in several murine models. It was especially active against an Ara-C resistant L1210 tumor. Inhibition of CTP synthesis was confirmed in mice bearing L1210 ascites. Four new cyclopentenyl nucleosides were synthesized and evaluated. Two pyrimidine nucleoside analogues, cyclopentenyl thymine and cyclopentenyl Ara-C, showed no inhibition of L1210 cell growth at concentrations of 0.1 mM. Cyclopentenyl nebularine inhibited L1210 cell growth by 65% at 10  $\mu$ M and cyclopentenyl 8-aza-adenosine inhibited by 33% at 2  $\mu$ M and 73% at 5  $\mu$ M. A method was developed to quantitate dihydrolenperone, a cytotoxic agent with lung tumor specificity. This analysis is being used in a Phase I-II pharmacokinetics study of this agent.



### Pharmacology of Cyclopentenyl Nucleosides

As an ongoing collaboration with the Medicinal Chemistry Laboratory of DTP, we have evaluated four new cyclopentenyl nucleosides synthesized by this group. Two pyrimidine nucleoside analogs, cyclopentenyl thymine and cyclopentenyl Ara-C, showed no inhibition of L1210 cell growth even at 0.1 mM. However, cyclopentenyl nebularine inhibited L1210 cell replication 65% at 10  $\mu$ M and cyclopentenyl 8-aza-adenosine inhibited by 33% at 2  $\mu$ M and 73% at 5  $\mu$ M. The latter compound exhibited modest anti-tumor activity against L1210 ascites in vivo. Further studies will extend this result as a larger amount can be synthesized.

**Pharmacology of Dihydrolenperone.** Dihydrolenperone, a cytotoxic agent with known specific activity against certain lung tumors, is presently in clinical trials. We have developed an HPLC method to quantify plasma levels of this drug and are using the method for pharmacologic studies in conjunction with the phase I studies of dihydrolenperone. The method uses a C3-reverse phase column and heptane sulfonic acid as an ion pairing agent dissolved in a 70:30 acetonitrile:water as the mobile phase. Dihydrolenperone is extracted from plasma using a basic extraction into ethylacetate. After removal of the solvent, the residue is dissolved in 0.1 N HCL and injected into the HPLC for analysis. The minimum detection level is 0.1 ng and the minimum level for quantification of dihydrolenperone is 1 ng/ml plasma by this method. Analysis of serial plasma samples obtained from two patients after oral administration of the dihydrolenperone, revealed no plasma levels greater than 2 ng/ml. We are presently waiting for dose escalation to a level where plasma concentrations are high enough to perform pharmacokinetic studies.

### Publications

1. Moyer, J.D., Malinowski, N.M., Treanor, S.P., and Marquez, V.E. Antitumor activity and biochemical effects of cyclopentenyl cytosine in mice. Cancer Res. 46: 3325-3329, 1986.
2. Kang, G.J., Cooney, D.A., Moyer, J.D., Kelley, J.A., Marquez, V.E., and Johns, D.G. Positive cooperative effect of cyclopentenyl cytosine triphosphate on bovine liver CTP synthesis. Biochem. Pharmacol. in press, 1987.
3. Hiraga, S., Klubes, P., Owens, E., Cysyk, R.L. and Blasberg, R.G. Brain tumor and cerebral blood flow is increased by blood-perfluorocarbon emulsion (fluosol-DA) exchange. Cancer Res. in press, 1987.
4. Klubes, P., Hiraga, S., Cysyk, R.L., Owens, E. and Blasberg, R.G. Attempts to increase intratumoral blood flow in the rat solid Walker 256 tumor by the use of the perfluorocarbon emulsion fluosol-DA. Eur. J. Cancer Clin. Oncol. in press, 1987.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECTPROJECT NUMBER  
Z01 CM 06163-03 LBC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Pharmacologic Aspects of Nucleotide Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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J. Strong	Pharmacologist	LBC, NCI
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2	1	1

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(a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inhibitors of nucleoside transport (e.g. dipyridamole) and inhibitors of uridine kinase were evaluated for effectiveness as inhibitors of uridine salvage both in vitro and in vivo for possible combination with inhibitors of de novo synthesis. Inhibitors of nucleoside transport were effective inhibitors of uridine salvage in vitro but not in vivo. Two inhibitors of uridine kinase (3-deazauridine and cyclopentenyl uracil) were found to be effective inhibitors of uridine salvage both in vitro and in vivo. Cyclopentenyl uracil has many advantages over other compounds tested and will be studied in depth when supplies of this compound become available. The contribution of urea cycle intermediates to de novo pyrimidine synthesis in the intact mouse was determined directly by infusion of 15NH4Cl and analysis of the incorporation of label and the distribution of label between the two nitrogens of the uracil ring by GC/MS. Both the liver and the small intestine incorporated the label directly into newly synthesized uracil nucleotides; however, the increase in newly synthesized uracil nucleotides in the kidney is due to increased salvage of uridine synthesized in the liver and output to the circulation. Precursors for de novo synthesis were evaluated for possible use in human studies. A suitable precursor is 15N-alanine which was found to adequately label the nucleotide pools of mouse bone marrow cells in the intact animal. Consequently, 15N-alanine will be used in human studies to quantitate de novo pathway activity during drug treatment.



Objective:

The overall objective of this project is to determine the relative dependency of host and tumorous tissues on de novo vs salvage pathways for the synthesis of pyrimidine and purine nucleotides in vivo. The de novo biosynthetic pathways supply pyrimidines and purines for nucleic acid synthesis and are therefore considered to be important pathways for cell proliferation. Therefore, there has been a considerable effort throughout the past several decades to develop specific inhibitors of enzymes of these pathways. Although a number of potent inhibitors (e.g. PALA, pyrazofurin, 6-azauridine) exhibit excellent in vitro activity against isolated enzymes and cultured cells and in vivo activity against certain murine tumors, only marginal clinical success has been achieved with these agents. It would appear that factors other than potency of inhibition are important because very potent enzyme inhibitors (PALA, pyrazofurin) are only marginally effective. Accordingly, lack of clinical success with the pathway inhibitors developed to date might be an indication that the importance of the de novo pathway to cell survival in vivo might be over-estimated. If so, then the development of additional inhibitors of the de novo pathway would be a futile effort unless there is a coordinate development of agents that either interfere with the salvage pathway or with the synthesis and export of preformed nucleosides by donor organ(s).

This Project is divided into the following specific aims: to determine the physiologic importance of circulating pyrimidines and purines and their role in modulating the antitumor activity of antypyrimidine and antipurine chemotherapeutic agents; to study the liver as a modulator of circulating nucleosides and as a possible target for chemical manipulation; to develop agents to interfere with nucleoside salvage to be used in combination with inhibitors of de novo synthesis; to develop methodology for monitoring and quantitating the flux through the de novo pathways of host and tumorous tissues in vivo.

Major Findings:

Development of Inhibitors of Nucleoside Salvage. The results discussed in previous annual reports of this project indicate that inhibition of nucleoside salvage could enhance the effectiveness of inhibitors of de novo nucleotide synthesis. We found that inhibitors of nucleoside transport, such as dipyridamole, have only limited effectiveness as inhibitors of nucleoside salvage in vivo. We found that two inhibitors of uridine kinase, 3-deazauridine and cyclopentenyl uracil, are effective inhibitors of uridine salvage both in vitro and in vivo. Cyclopentenyl uracil inhibits uridine kinase with a  $K_i$  value of 0.23 mM. This nucleoside does not inhibit the growth of cultured L1210 cells even at a concentration of 0.5 mM, but inhibits the salvage of uridine by these cells by 58% at a concentration of 0.1 mM cyclopentenyl uracil. This compound also inhibits the salvage of cytidine, but not that of deoxycytidine or thymidine, which is consistent with inhibition of the kinase as the site of action. PALA, as inhibitor of the de novo pathway, inhibits the growth of cultured L1210 cells. This inhibition is reversed by concentrations of uridine (1-10 micromolar) similar to that found in the plasma in vivo. The addition of cyclopentenyl uracil (300  $\mu$ M) restores the sensitivity of PALA. For example, cells grown in medium containing 1 mM PALA grow at the same rate as controls if the medium contains 10  $\mu$ M uridine in addition to the PALA. If cyclopentenyl uracil (300  $\mu$ M) is also present, cell growth is



inhibited by 67%. Thus in this model cyclopentenyl uracil is highly synergistic with PALA, and in fact, converts it from an inactive to an active agent. These results encouraged us to evaluate the ability of cyclopentenyl uracil to inhibit nucleoside salvage *in vivo* in the intact mouse. Our results indicate that cyclopentenyl uracil can inhibit uridine salvage by various tissues and ascites L1210 by 65-95% as measured at 2 h after a dose of 1 gm/kg. We found that the duration of action is rather short and thus schedules of 8-12 h injections are necessary for a continued inhibition of salvage. Future experiments will determine the feasibility of combinations of nucleoside salvage inhibitors and de novo pathway inhibitors as antitumor therapy.

Contribution of Urea Cycle Intermediates to De Novo Pyrimidine Synthesis. We reported in earlier annual reports that ammonia stimulates de novo pyrimidine synthesis in hepatocytes, liver, and in the intact animal. In the current study  $^{15}\text{NH}_4\text{Cl}$  was infused into mice at varying doses and the incorporation of label into the uracil nucleotide pools and urea determined by GC/MS analysis of extracts of liver, small intestine, and kidney. Analysis of the distribution of label between the two nitrogens in the uracil ring allows us to calculate the fraction of the uracil nucleotide pool formed by de novo synthesis during the labeling period. When the dose of  $^{15}\text{NH}_4\text{Cl}$  was increased from 50 moles/hr to 250 moles/h the fraction of the total uracil nucleotide pool formed by de novo synthesis increased 4.0-fold in liver to 150 nmoles/hr/g and 2.3-fold in intestine to 137 nmoles/hr/g. The increase in intestine was independent of the increase in liver as evidenced by the lack of correlation between the increases observed in the intestine and liver of the same animal and the different distributions of label in the uracil ring nitrogens. A 2.4-fold increase in newly formed uracil nucleotides was observed in kidney when the infusion dose was raised from 150 moles/hr to 250 moles/hr. The increase in kidney was correlated with the increase in liver in the same animal and the distribution of label in the uracil ring nitrogens was similar to the distribution of label in liver. These results suggest that the increase in newly formed uracil nucleotides in kidney is due to increased salvage of uridine synthesized in the liver and output to the circulation. Thus the contribution of intermediates of the urea cycle to de novo pyrimidine synthesis may be substantial in hepatic and extra-hepatic tissues.

#### De Novo Pyrimidine Synthesis in Humans

Recently, we have started a collaboration with the Clinical Oncology Branch to help evaluate the effect of methotrexate on immature bone marrow cells. The objective of this study is to evaluate the effect of methotrexate on flux through the de novo purine biosynthetic pathway in these cells *in vivo* in human studies. We plan to use the stable isotope methods developed in our laboratory in combination with  $[^{15}\text{N}]$ -alanine as a precursor. The success of this project depends on the sensitivity of our techniques and we are presently testing the feasibility of this study in mouse bone marrow and with human bone marrow *in vitro*. The initial data we have collected looks positive. In one experiment mice were infused for 1 h with  $[^{15}\text{N}]$ -alanine and bone marrow, collected, pooled, and immature cells isolated. The cells were then analysed for  $^{15}\text{N}$ -enrichment using our GC/MS method. The enrichment measured was 1-2 mole % and the precision of the measurement was acceptable.



Publications

1. Karle, J.M., Chisena, C., Cowan, K.H. and Cysyk, R.L.: Uracil nucleotide synthesis is a human breast cancer cell line (MC-7) and two drug resistant sublines that contain increased levels of enzymes of the de novo pyrimidine pathway. J. Mol. Pharm., 30: 136-141, 1986.
2. Arnold, S.T. and Cysyk, R.L. Adenosine export from the liver: oxygen dependency. Am. J. Physiol., 251: G34-G39, 1986.
3. Klubes, P., Geffen, D.B. and Cysyk, R.L. Comparison of the bioavailability of uridine in mice after either oral or parenteral administration. Cancer Chem. and Pharm., 17: 236-240, 1986.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06164-03 LBC

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inhibitors of Phospholipid Metabolism as Potential Chemotherapeutic Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biological Chemistry

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Agents which block the formation of second messengers that mediate growth factor action may be of value in cancer chemotherapy. Recently the hydrolysis of phosphatidylinositol-diphosphate to produce diacylglycerol (DAG) and inositol triphosphate has been implicated in the action of a number of growth factors including platelet derived growth factor and bombesin. We are therefore attempting to examine the chemotherapeutic effects of inhibition of this process and attempting to develop inhibitors of number of the enzymes involved in PI metabolism. During this period we have published a new method for the separation and measurement of inositol phosphates. This technique revealed that the metabolism of these compounds is more complex than previously known and involves multiple positional isomers. Subsequent investigation has revealed that in homogenates of GH3 rat pituitary cells inositol triphosphate is catabolized primarily to the inositol (1,3,4) triphosphate. This triphosphate then is further catabolized to a mixture of the 1,3- and 3,4-bisphosphates. The inositol (1,4) bisphosphate is catabolized exclusively to the 4-monophosphate by homogenates of these cells. We have partially purified an enzyme from rat liver which dephosphorylates inositol (1,3,4) triphosphate, but none of the other 6 inositol phosphates examined. Studies in collaboration with Dr. E. Sausville of Naval Med. Center have established that exposure of small cell lung cancer cells to bombesin produces an increase in several of the inositol phosphates, implicating hydrolysis of PIP<sub>2</sub> in the action of this growth factor on these cells. Further studies are in progress to elucidate the role of PI metabolism in growth regulation. Our initial efforts in drug development have centered on myo-inositol analogs. In collaboration with Dr. D. Baker at the U. of Alabama we have published a synthesis of 14 analogs and have examined their structure activity relationship as inhibitors and substrates of PI synthetase. Several of these analogs show potential for further development.



Objectives.

Reduced requirements for stimulation by growth factors may be the fundamental characteristic of transformed (neoplastic) cells. Research in molecular biology has identified several specific biochemical changes produced by introduction or overexpression of oncogenes which may reduce the levels of exogenous growth factors needed to trigger cell replication. These findings suggest that a more selective approach to chemotherapy may focus on the interaction of growth factors with cells rather than on basic metabolic reactions such as those involved in nucleic acid synthesis. We have therefore initiated a project to develop new chemotherapeutic agents to block the action of growth factors.

Recent findings indicate that inositol-phosphates formed from phosphatidyl-inositides may be the second-messengers which mediate the action of many growth factors. In addition, numerous alterations in phosphoinositide metabolism have been associated with transformation with specific oncogenes. The synthesis of phosphatidylinositol polyphosphates and their subsequent hydrolysis therefore provide attractive well-defined targets for the design of inhibitors for use in chemotherapy. This project has two related goals. First, to determine the relative importance of the enzymatic reactions involved in inositol phosphate production as a signal initiating cell replication. Secondly, to design, synthesize, and evaluate drugs to inhibit this process, specifically inhibitors of the reactions of phosphatidylinositol polyphosphate synthesis and of phospholipase C, the enzyme which produces the inositol phosphates.

Major Findings.

1. The metabolism of inositol phosphates by homogenates of GH3 rat pituitary cells has been characterized in detail. We found that IP<sub>4</sub> is metabolized to inositol (1,3,4)-triphosphate and then to inositol (1,3)-bisphosphate and inositol (3,4)-bisphosphate. Inositol (1,4)-bisphosphate is dephosphorylated exclusively at the 1-position by a highly specific phosphatase of 58,000 MW with a K<sub>m</sub> of 0.8  $\mu$ M.
2. In collaboration with Dr. E. Sausville of Naval Medical Center we have examined the response of human small cell lung cancer cells to the growth factor bombesin. These cells respond to bombesin with a rapid increase in the levels of inositol (1,4,5)-triphosphate and inositol (1,3,4)-triphosphate and a slower accumulation of inositol monophosphate. The response to bombesin exhibits the same structure activity relationship as the mitogenic response and the accumulation of inositol phosphates is ablated by pretreatment of the cells with phorbol esters as is the transient increase in intracellular Ca<sup>++</sup> induced by bombesin. One line of non small cell lung cancer cells did not exhibit an increase in inositol phosphates upon exposure to bombesin or EGF.
3. Murine L1210 cells were shown to grow without addition of myo-inositol to the medium, and to synthesize myo-inositol from glucose. This synthesis as well as the intracellular levels of myo-inositol were determined by a mass-spectrometric method in a collaboration with Dr. J. Strong of this laboratory.
4. We published the synthesis and evaluation of a series of analogs of myo-inositol and "scaled up" the synthesis of the most active analog for further evaluation.



Proposed Course.

1. The metabolism and effects of the newly synthesized inositol analogs will be further explored to determine if they effectively inhibit PI turnover in intact cells. 2. New inhibitors of phospholipase C and PI kinase will be synthesized and evaluated. 3. The metabolism of phosphoinositides in small cell lung cancer cells will be further characterized. In particular we will examine the activation of protein kinase C, the formation of diacylglycerol and differences between "classic" and varient forms of small cell lung cancer cells.

Publications.

Dean, N.M., and Moyer, J.D. Separation of multiple isomers of inositol phosphates formed in GH3 cells. Biochem. J. 242: 361-367, 1987.

Moyer, J.D., Reizes, O., Jiang, C., and Baker, D.C. A new approach to the inhibition of growth factor action. Analogs of myo-inositol. Proc. Am. Assoc. Cancer Res. 28: 307, 1987.

Jiang, C., Moyer, J.D., and Baker, D.C. Synthesis of deoxy- and deoxyhalogene analogs of myo-inositol. J. Carbohydrate Chem., In Press, 1987.

Cunha-Melo, J.R., Dean, N., Moyer, J.D., Maeyama, K., and Beaven, M.A. The pattern of phosphoinositide hydrolysis in rat basophilic leukemia (RBL-2H3) cells varies with the type of IgE receptor cross-linking reagent used. Biol. Chem., In Press, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06166-03 LBC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Mechanism of Multidrug Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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## CHECK APPROPRIATE BOX(ES)

(a) Human subjects  (b) Human tissues  (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Radioactive photoactive drug analogues have been used to identify specific drug binding targets in multidrug-resistant cells. A 150-180 kDalt surface membrane glycoprotein (P-gp) which binds Vinca alkaloids has been identified in "classic" but not "atypical" multidrug resistant cell lines by photolabeling with a photoactive analogue of vinblastine. P-gp exhibits a cross-specificity for several natural product drugs suggesting that it has a functional role in determining the multidrug-resistant phenotype. The involvement of P-gp in the circumvention of drug-resistance by calcium channel blockers was evaluated by comparing the ability of various blockers to interfere with P-gp vinblastine photolabeling and to stimulate drug efflux and increase drug-accumulation. This data plus the ability to photoaffinity label P-gp with a photoactive analogue of a dehydropyridine calcium channel blocker indicates that P-gp plays a direct role in the mechanism of reversal of multidrug resistance by calcium channel blockers. A functional P-gp will be purified from multidrug-resistant cells and characterized with respect to drug binding. Purified P-gp will be inserted into artificial lipid vesicles for functional reconstitution studies. P-gp specific drug analogues with alkylating activities will be synthesized and used to block drug uptake, efflux and cellular accumulation in multidrug-resistant cells. The new drug analogues will be tested for their ability to circumvent multidrug-resistance in vivo.



The exposure of malignant cell lines to natural product cytotoxic drugs such as vinblastine, actinomycin D, adriamycin or colchicine frequently results in the isolation of populations of cells with resistance to the selecting agent as well as a collateral resistance to other mechanistically distinct and structurally unrelated compounds. The mechanism(s) by which these cell lines become multi-drug-resistant is unknown, but it is thought to be related to a parallel reduction in the cellular accumulation of those drugs to which the cells are resistant. The multidrug-resistant phenotype also is characterized by the presence of a 150-180 kDa surface membrane glycoprotein P-gp which occurs in multidrug-resistant cells in direct proportion to the degree of their acquired drug-resistance. The relationship of P-gp to multi-drug resistance is not known. It may accumulate only as a secondary consequence of the multidrug-resistant phenotype. Alternatively, P-gp could promote multi-drug resistance by direct or indirect effects on membrane permeability, drug transport, or drug binding.

#### Objectives.

This project involves the identification of specific drug interactions with macromolecules in normal and multidrug-resistant cell lines. The relationship of specific drug binding macromolecules to multidrug-resistance mechanisms will be examined. New chemotherapeutic agents designed to circumvent multidrug-resistance will be designed, synthesized and tested.

#### Major Findings.

Identification of Vinca alkaloid acceptors in P388 murine leukemia cells with a photoactive analog of vinblastine. The cytotoxic, antimitotic and growth inhibition properties of a photoactive analog of vinblastine, N-(p-azidobenzoyl)-N'-β-aminoethylvinidesine (NABV) and vinblastine on P388 murine leukemia cells were compared. After 72 h exposure, the IC<sub>50</sub> values of exponentially growing P388 leukemic cells were 1.2 nM for NABV and 0.6 nM for vinblastine. The ultrastructural effects of NABV and vinblastine on P388 cells were similar: formation of microtubular crystals; mitotic arrest (C-mitosis); increased post C-mitotic multinucleated cells; increased number of annulated lamellae; the appearance of intracytoplasmic paired cisternae. [<sup>3</sup>H]NABV was used to identify Vinca alkaloid binding sites in P388 cells by photoaffinity labeling. After irradiation at 302 nm, radioactive Vinca alkaloid binding components were resolved by SDS-PAGE and identified in 1 mm gel slices. The most prominent photolabeled species were Mr 44,000, 54,000 and 75,000 polypeptides located in the 100,000 x g supernatant fraction. The 54,000 component was also observed in the membrane fraction. Specific photolabeling of Mr 54,000 and 44,000 polypeptides was blocked in the presence of 200-fold excess of vinblastine and was saturable with half maximum saturation concentrations of 0.6 and 0.4  $\mu$ M [<sup>3</sup>H]NABV, respectively. The Mr 54,000 component was identified as a tubulin subunit by immunoprecipitation with antitubulin monoclonal antibodies. Since NABV and vinblastine have similar pharmacological and biological properties, this photoactive analog may be useful for identifying important Vinca alkaloid cellular acceptors which may be responsible for drug cytotoxic and antineoplastic activities.



Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. A radioactive photoactive dihydropyridine calcium channel blocker, [<sup>3</sup>H]azidopine, was used to photoaffinity label plasma membranes of multidrug-resistant Chinese hamster lung cells selected for resistance to vincristine (DC-3F/VCrd-5L) or actinomycin D (DC-3F/ADX). Sodium dodecyl sulfate polyacrylamide gel electrophoretic fluorograms revealed the presence of an intensely radiolabeled 150-180 kDa doublet in the membranes from drug-resistant but not from the drug-sensitive parental (DC-3F) cells. A similar radiolabeled doublet was barely detected in a drug-sensitive partial revertant (DC-3F/ADX-U) cell line. The 150-180 kDa doublet exhibited a specific half-maximal saturable photolabeling at  $1.07 \times 10^{-7}$  M [<sup>3</sup>H]azidopine. The dihydropyridine binding specificity was established by competitive blocking of specific photolabeling with nonradioactive azidopine as well as with nonphotoactive calcium channel blockers nimodipine, nitrendipine and nifedipine. In addition, [<sup>3</sup>H]azidopine photolabeling was blocked by verapamil and diltiazem but was stimulated by excess prenylamine and bepridil suggesting a cross-specificity for up to 4 different classes of calcium channel blockers. The 150-180 kDa calcium channel blocker acceptor co-electrophoresed exactly with the 150-180 kDa surface membrane glycoprotein (P-gp) Vinca alkaloid acceptor from multidrug-resistant cells and was immunoprecipitated by polyclonal antibody recognizing P-gp. [<sup>3</sup>H]Azidopine photolabeling of the 150-180 kDa component in the presence of excess vinblastine was reduced over 90%, confirming the identity or close relationship of the calcium channel blocker acceptor and the P-gp Vinca alkaloid acceptor. The [<sup>3</sup>H]azidopine photolabeling of gp150-180 also was reduced by excess actinomycin D, adriamycin, or colchicine, demonstrating a broad P-gp drug recognition capacity. The ability of P-gp to recognize multiple natural product cytotoxic drugs as well as calcium channel blockers suggests a direct function for P-gp in the multidrug resistance phenomenon and a role in the circumvention of that resistance by calcium channel blockers.

Comparison of the biochemistry and cellular pharmacology of "atypical" and "classic" multidrug resistance. We have selected a human leukemic cell line for resistance to the epipodophyllotoxin, teniposide (VM-26). This line, CEM/VM-1, displays "atypical" multidrug resistance (at-MDR) in that it is cross-resistant to a wide variety of natural product antitumor drugs but not to the Vinca alkaloids. Consistent with this alkaloid sensitivity, these cells accumulated as much [<sup>3</sup>H]vincristine at steady-state as did the drug-sensitive parent cells. However, this VM-resistant cell line also accumulated as much labeled epipodophyllotoxin as did the parent line. "Classic" MDR cells, such as CEM/VLB100, exhibit decreased drug accumulation and over express a prominent glycoprotein of approximately 170 to 190 kDa (known as P170, gp180 or P-glycoprotein), compared to the drug-sensitive parent cells. Labeling of external plasma membranes of CEM/VM-1 cells with [<sup>3</sup>H]borohydride revealed some differences in surface glycoproteins compared to both drug-sensitive and classic MDR cells, but specific labeling of P-glycoprotein was not seen. This observation was substantiated by the inability to label this MDR-associated protein with the photoaffinity probe, N(p-azido-[3-<sup>125</sup>I]salicyl)-N'- $\beta$ -aminoethylvinidesine. Furthermore, no hybridization of the pMDR1 cDNA was seen in slot-blot analyses of the RNA from at-MDR cells, indicating that the mdr gene coding for P-glycoprotein is not overexpressed as is the case in the classic MDR cells. However, karyotype analysis indicated that the CEM/MV-1 cells contained an abnormally banded region on chromosome 13q, providing cytologic



evidence for amplification of a gene other than mdr in these cells. Thus, despite the two cell lines having approximately equal degrees of resistance to epipodophyllotoxins, our data indicate that the mechanism(s) responsible for at-MDR is different than that for classic, p-glycoprotein-associated MDR.

Proposed Course.

The above data confirm the usefulness for identifying drug binding proteins by photoaffinity labeling. A major P-gp Vinca alkaloid acceptor has been identified in multidrug-resistant cells. Other drugs will be used to establish the multidrug specificity of P-gp. The role of P-gp in drug resistance will be examined using agents previously shown to counteract multidrug resistance. The effect of photolabeling on cellular drug uptake and efflux will be examined. The subcellular distribution of gp150-180 with time will be monitored. The P-gp will be purified using immunoabsorption and affinity chromatographic methods. Partial structural analysis will be used to identify drug binding sites. Ultimately, gp150-180 will be inserted into artificial lipid vesicles for functional reconstitution experiments. From this understanding of the multidrug-resistance mechanism, we will synthesize and test new compounds designed to reverse the multidrug-resistant state.

Publications:

1. Averbuch, S.D., Clawson, R.E., Bachur, N.R. and Felsted, R.L. Cellular pharmacology and antitumor activity of N-(p-azidobenzoyl)daunorubicin, a photoactive anthracycline analogue. Cancer Chemother. Pharmacol., 16: 211-217, 1986.
2. Safa, A.R., Glover, C.J., Meyers, M.B. Biedler, J.L. and Felsted, R.L. Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. J. Biol. Chem., 261: 211-217, 1986.
3. Averbuch, S.D., Glover, C.J. and Felsted, R.L. Anthracycline photoaffinity labeling of a mitochondrial polypeptide in P388 murine leukemic cell lines. Cancer Res., 46: 6120-6124, 1986.
4. Safa, A.R. and Felsted, R.L. Specific Vinca alkaloid binding polypeptide identified in calf brain by photoaffinity labeling. J. Biol. Chem., 262: 1261-1267, 1987.
5. Felsted, R.L., Glover, C.J., Clawson, R.E. and Averbuch, S.D. Rat heart anthracycline binding polypeptides identified by photoaffinity labeling. Mol. Pharmacol., 30: 388-397, 1986.
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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06167-03 LBC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inhib. of Myristoylation-Dependent Cell Transformation & Retroviral Replication

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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2.5	1.5	1

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<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The modification of onc-proteins with the fatty acid myristate is an early step associated with the transformation of normal to neoplastic cells and mammalian retroviral reproduction. The exact significance of myristylation in transformation has not been established. However, it is thought to be part of the mechanism by which cytoplasmic oncogene or viral gag structural protein. Kinases are localized to the inner plasma membrane surface. Since the transforming activity of onc-kinases and viral replication is dependent upon this membrane binding, this project will investigate the role of myristylation as it relates to the mechanism of this subcellular localization. A specific myristylation assay has been developed using synthetic NH<sub>2</sub>-terminal polypeptide homologues of cellular and viral myristoylated proteins. The myristoyl transferase(s) substrate specificity for polypeptide and fatty acid will be examined in order to define the myristylation mechanism. The involvement of myristic acid in membrane binding will be examined by looking for specific membrane receptors of the myristoyl-proteins. Information on the enzymology of myristylation and the role of myristic acid in membrane binding will be used to design and synthesize specific inhibitors of myristylation and membrane association with the goal of developing chemotherapeutic agents specific for critical early steps of tyrosine kinase mediated malignant transformation and mammalian retroviral reproduction.



Tyrosine-specific protein kinase activity is associated with several known oncogenes and is an appealing target for the chemical manipulation of kinase associated cellular transformation. The transforming activity of onc-kinases depends upon their association with the inner plasma membrane surface. It has been proposed that the mechanism by which viral encoded onc-kinases such as p60<sup>src</sup> become membrane bound is through a post- or co-translational addition of myristic acid to their NH<sub>2</sub>-terminal glycine via an amide linkage. Myristoylation is also an essential step in the replication of type B,C & D retroviruses and is typified by the covalent attachment of myristic acid to the NH<sub>2</sub>-terminal glycine of viral p24<sup>gag</sup> structural proteins via an amide linkage. These myristoylated gag proteins are then localized to the inner plasma membrane where viral assembly and maturation occurs. The importance of myristoylation to onc kinase transformation and retroviral replication has been illustrated by oligonucleotide-directed mutagenesis, resulting in mutant gene proteins in which NH<sub>2</sub>-terminal glycines were either absent or replaced with alanine. These mutant proteins are no longer localized to the membrane but are found in the cytoplasm. Most pertinent to this proposal, these mutant proteins no longer transformed cells nor were viral particles formed. Presumably, myristoylation is critical to onc-kinase transformation and retroviral reproduction.

### Objectives.

In this project we will examine the role of myristoylation of onc-tyrosine kinases and retroviral gag structural proteins as targets for the chemotherapeutic inhibition of cellular transformation and viral reproduction. Specifically, we will study two mechanisms for blocking myristoylation dependent transformation by p60<sup>src</sup>: these include, (i) inhibition of the myristoyl transferase(s), and (iii) inhibition of binding of myristoyl-proteins to potential membrane receptors.

### Major Findings.

N-Myristoylation of p60<sup>src</sup>: Identification of myristoyl CoA glycylpeptide N-myristoyl transferase in rat tissue. A 16 residue synthetic peptide corresponding to the NH<sub>2</sub>-terminal sequence of p60<sup>src</sup> was used as the acyl acceptor in an assay for myristoyl CoA:glycylpeptide N-myristoyl transferase in rat tissues. An additional COOH-terminal tyrosine amide was added to this peptide to facilitate radioiodination and enhance detectability. Reverse phase HPLC enabled the simultaneous detection and quantitation of the peptide substrate and its N-myristoylated product. N-Myristoyl transferase activity was highest in the brain with decreasing activities in lung, small intestine, kidney, heart, skeletal muscle, and liver. Brain activity was distributed approximately equally between the 100,000 x g pellet and supernatant fractions. The soluble enzyme exhibited a  $K_m^{app}$  of 20  $\mu$ M for the src peptide and an optimum between pH 7-7.5. Maximum N-acylating activity was seen with myristoyl (C14:0) CoA with lower activities found with the C10:0 CoA and C12:0 CoA homologues. No activity was obtained with palmitoyl (C18:0) CoA but this derivative inhibited N-myristoyl transferase activity >50% at equal molar concentrations with myristoyl CoA. With a decapeptide corresponding to the NH<sub>2</sub>-terminal sequence of the cAMP dependent protein kinase catalytic subunit as the acyl acceptor, the brain enzyme displayed a  $K_m^{app}$  of 117  $\mu$ M and was about 15x less



catalytically effective than the p60<sup>src</sup> acyl acceptor. Inhibition studies with shorter src peptide analogues indicated an enzyme specificity for the p60<sup>src</sup> acyl acceptor beyond 9 residues.

#### Proposed Course

Myristoylation is thought to be an important cellular phenomenon. The fact that such a rare fatty acid is utilized with such absolute specificity by cellular enzymes with broad regulatory effects as well as by transforming onc-kinases suggest it has a central role in the control of cellular growth and differentiation. Its involvement in mammalian retrovirus replication utilizes the basic myristoylation pathways. The projects outlined in this proposal are designed to clarify several specific aspects concerning the process of myristoylation. From this work, it may be possible to design novel new compounds for specifically blocking myristoylation. These agents may then provide us with pharmacologic tools to specifically inhibit myristoylation dependent cellular transformation and mammalian retroviral related disorders.

At least two key aspects of myristoylation dependent membrane binding of transforming oncogene and retroviral gag structural proteins may be susceptible to direct chemotherapeutic manipulation. These include, (i) inhibition of the myristoyl transferase(s), and (ii) inhibition of binding of myristoyl-proteins to potential membrane receptors. Most of the current work on the myristoylation phenomena is directed toward the identification of new myristoyl-proteins and the defining of their fatty acid attachment site amino acid sequences. Little is known about myristoyl transferase(s) nor is there any information about hypothetical membrane receptor sites. We would predict that both steps are essential to the overall myristic acid dependent transforming mechanism and therefore, each represents an opportunity to block malignancy that results from this type of transformation and viral reproduction.

(i) N-Myristoyl Transferase(s). We are continuing our characterization of mammalian myristoyl transferases using peptide acceptors corresponding to the NH<sub>2</sub>-terminal sequence of p60<sup>src</sup>, p24<sup>gag</sup> and cAMP dependent protein kinase. The tissue distribution and substrate specificities of the transferases is being studied. The enzyme(s) will be purified and the enzymatic mechanism characterized. We will design specific, irreversible and competitive inhibitors of the enzyme. These will be synthesized and tested as inhibitors of oncogene-kinase mediated transformation and retroviral replication in tissue culture cells.

(ii) Myristoyl Protein Membrane Binding. Membrane binding sites for myristoyl proteins will be investigated by classical receptor techniques. Using nonradioactive and [<sup>125</sup>I]peptides, we will look for functional p60<sup>src</sup> binding sites in platelets and transformed cell lines. Myristoyl protein receptors will be identified with photoactive peptide analogues and purified by affinity absorption to peptide affinity resins. Specific agents will be designed to block myristoyl protein-receptor association. These will be tested as inhibitors of oncogene-kinase mediated transformation and/or retroviral replication in tissue culture cells.



Publications:

1. Glover, C.J., Goddard, C. and Felsted, R.L. Myristylation of p60<sup>src</sup>: Identification and initial characterization of N-myristoylglycyl-peptide transferase activities in rat tissues. Proc. Am. Assoc. Cancer Res. 28: 4, 1987.



## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Role of Protein Kinase C &amp; Tyrosine Kinase in Differentiation and Drug Resistance

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(a) Human subjects  (b) Human tissues  (c) Neither

(a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Calcium- and phospholipid-dependent protein kinase (PK-C) activity was determined in association with the differentiation response of HL-60 leukemia cells to phorbol ester (TPA). Conditions under which TPA produced the macrophage phenotype resulted in disappearance of PK-C, but duplication of this phenotypic response with one hour priming doses of TPA and post treatment with retinoic acid showed conclusively that down regulation of PK-C is not a necessary consequence of the macrophage phenotype. Coincident with the appearance of the macrophage phenotype is the phosphorylation of vinculin *in vitro*. This phenomenon is also present in Adriamycin-resistant HL-60 cells as well as in other multidrug resistant cells. The regulation of phosphorylation of this actin-binding protein is being investigated with antibodies to protein kinase C. A new investigation has been initiated to study the regulation of plasma membrane-associated tyrosine protein kinase (p98) in HL-60 leukemia cells undergoing differentiation and in cells resistant to differentiating agents. A non-denaturing gel electrophoretic assay for multiple tyrosine kinase activities in cell extracts has been developed, and this assay has facilitated the purification of p98. The possible association between p98 and the receptor for granulocyte-macrophage colony stimulating factor and its identity as the cellular *fps* gene product is being investigated.



The role of protein kinases in the regulation of cell proliferation and differentiation is receiving increased emphasis within the framework of recent advances in protooncogene function and hormone and growth factor receptors. The calcium- and phospholipid-dependent protein kinase (protein kinase C, PK-C) which is ubiquitous within the animal kingdom is believed to serve a crucial regulatory role in mediating or modulating the action of growth factors. For example, phosphorylation of EGF receptor by PK-C reduces the receptors affinity for EGF. The human promyelocytic leukemia cell line HL-60 contains an abundance of PK-C. We have recently documented increased PK-C in response to granulocytic or monocytic differentiating agents such as DMSO, retinoic acid and 1,25-dihydroxyvitamin D<sub>3</sub>. Interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor (TNF), both discovered in our lab to be monocytic differentiating agents, also induce elevated PK-C activity in the mature phenotype. The response of PK-C to these agents appears to be differentiation-related and serves as a good marker of the mature cell type. Associated with the appearance of the macrophage phenotype is the phosphorylation *in vitro* of the actin-binding protein, vinculin. This phenomenon occurs to an even greater extent in Adriamycin-resistant HL-60 cells which coincidentally are resistant to all differentiating agents except TPA. The phosphorylation of vinculin *in vitro* as well as *in vivo* occurs in all multi-drug resistant cells examined thus far including MCF-7 and Chinese hamster lung cells. We believe that this process is associated with the calcium-dependent proteolytic processing of protein kinase C to the Ca<sup>++</sup>- and phospholipid-independent form termed M-kinase. We plan to use antibodies against purified bovine brain protein kinase C as well as a synthetic peptide to study this phenomenon. In addition, Ca<sup>++</sup>-dependent protease activity, which is responsible for the formation of M-kinase, will be investigated in multi-drug resistant cells.

An ancillary area of research involves our recent discovery of a plasma membrane-bound tyrosine protein kinase that is induced during the differentiation of HL-60 cells by several granulocytic (DMSO, retinoic acid) and monocytic (1,25-dihydroxyvitamin D<sub>3</sub>, tetradecanoyl phorbol acetate (TPA), IFN- $\gamma$  and TNF) differentiating agents. This tyrosine protein kinase appears within 1 day after treatment of HL-60 cells with IFN- $\gamma$  or TNF and is elevated in proportion to the degree of maturation of the cells. The development of a non-denaturing polyacrylamide gel electrophoretic assay system has allowed us to characterize the various species of tyrosine kinase activities in crude cytoplasmic and membrane preparations in a variety of cell lines including drug resistant cells. We have recently found in collaboration with Steven Grant, Columbia University, that HL-60 cells that are 100-fold resistant to Adriamycin are cross resistant to several differentiating agents and are devoid of the membrane-bound tyrosine kinase found in HL-60 cells sensitive to these agents. This tyrosine protein kinase has recently been purified in our lab and is termed p98. It is related to the viral oncogene *v-fps/fes*, the cellular counterpart of which is associated with stem cells in the bone marrow of chickens. We have recently produced evidence that p98 is the cellular receptor for granulocyte-macrophage colony stimulating factor (GM-CSF) using recombinant GM-CSF. Further studies to sequence the N-terminus of p98 are planned so that the protein may be cloned.

#### Major Findings

1. The ability of retinoic acid (RA) to promote TPA-initiated macrophage



differentiation was examined in human promyelocytic leukemia cell line HL-60. One hr exposure to 10 nM TPA and subsequent exposure for 48 hr to 1  $\mu$ M RA following removal of TPA rapidly induced the macrophage phenotype in 65% of the cells. This effect was comparable to continuous exposure for 48 hr to TPA alone, but contrasted with the absence of macrophage-like cells after RA treatment alone or the induction of 10% of the cell population to a macrophage phenotype after 1 hr exposure to TPA. The effect of TPA + RA was accompanied by increased cell adherence and increased non-specific esterase activity but not by a change in the reduction of nitroblue tetrazolium. Protein kinase C (PK-C) activity was increased 35-40% in cells treated for 1 hr with TPA alone or after subsequent exposure to RA. Cells treated for 48 hr with RA exhibited a 2-fold increase in PK-C activity while cells exposed to TPA for 48 hr lost all PK-C activity. The changes in PK-C activity in TPA + RA-treated cells were accompanied by phospholipid- and  $Ca^{++}$ -dependent phosphorylation *in vitro* of pp38 which is characteristic of treatment with RA alone, as well as the phospholipid- and  $Ca^{++}$ -independent phosphorylation *in vitro* of pp82 and pp130 (vinculin) which is prevalent in cells treated continuously with TPA alone and is absent in RA-treated cells. These results indicate that the macrophage phenotype induced by TPA + RA is similar to that produced by continuous exposure to TPA alone with respect to their *in vitro* phosphoprotein patterns, cytochemical markers, cell adherence and morphology, but that the disappearance of PK-C is not an obligatory characteristic of these cells.

2. The effect of the combination of RA and calcium ionophore A23187 on cellular differentiation was assessed in promyelocytic leukemia cell line HL-60. RA at  $10^{-10}$  to  $2.5 \times 10^{-8}$  M or  $4 \times 10^{-7}$  M A23187 produced a 15 to 22% increase in differentiated cells reducing nitroblue tetrazolium. Exposure of cells for 48 h to the combination of  $4 \times 10^{-7}$  M A23187 and  $10^{-10}$  to  $2.5 \times 10^{-8}$  M RA resulted in a 20 to 86% increase in cells reducing nitroblue tetrazolium, but with no measurable level of nonspecific esterase activity. Cotreatment of these cells with A23187 and either DMSO, 1,25-dihydroxyvitamin D<sub>3</sub> or human immune IFN- $\gamma$  failed to produce a synergistic effect on differentiation. Addition of either the calmodulin antagonists, N-(6-aminohexy)-5-chloronaphthalenesulfonamide (W7) and trifluoperazine, or the protein kinase C inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) during treatment with A23187 and RA did not block differentiation. Membrane tyrosine kinase activity was measured in cells treated with A23187 and RA in a nondenaturing gel system using the exogenous substrate poly(Glu:Tyr). Membrane-bound tyrosine kinase activity was not present in untreated or RA-treated cells, but was induced by A23187 treatment alone and was markedly increased in cells after 48 h treatment by the combination of A23187 and RA. Significant reduction in c-myc mRNA levels was also observed after 24 h treatment of HL-60 cells with RA and A23187 but not by either agent alone. These results suggest that a  $Ca^{2+}$  mediated process sensitizes cells to the differentiating effect of RA and that this effect is associated with a significant reduction of c-myc expression and the induction of membrane tyrosine kinase activity in this cell line.
3. The effect of IFN- $\gamma$  and recombinant tumor necrosis factor (rTNF- $\alpha$ ) on cellular differentiation was investigated in human promyelocytic leukemia cell line HL-60. Both IFN- $\gamma$  and rTNF- $\alpha$  induced the appearance of the monocytic phenotype in a dose- and time-dependent manner as assessed by



morphology, reduction of nitroblue tetrazolium and the induction of non-specific esterase. Utilizing a nondenaturing polyacrylamide gel electrophoretic assay, it was revealed that a membrane-bound tyrosine kinase activity accompanied the appearance of the differentiated cell type. In contrast, other tyrosine kinase activities were either unaltered or reduced during differentiation. These results suggest that the induction of membrane-bound tyrosine kinase by IFN- $\gamma$  and rTNF- $\alpha$  may be an important determinant of the differentiation process.

4. A general procedure is presented for detecting tyrosine kinase activity in crude or purified preparations using nondenaturing gel electrophoresis. Samples are resolved by electrophoresis in mini gels which are then incubated in an assay mixture containing [ $\gamma^{32}\text{P}$ ]ATP, poly(glutamic acid, tyrosine)4:1 and cofactors. Subsequently, gels are fixed and washed in trichloroacetic acid-pyrophosphate, dried and analysed by autoradiography or liquid scintillation counting. The procedure is simple and fast and allows analysis of different molecular weight forms of tyrosine kinase under linear kinetics at 37° without interference from phosphatases and proteases.
5. Two tyrosine protein kinase activities have been identified previously to be present in HL-60 leukemia cells during induction of granulocytic and monocytic differentiation with a variety of differentiating agents. We have co-purified a membrane-associated tyrosine protein kinase (p98) and an activity associated with both the cytosol and membrane fractions (p60) based on their ability to phosphorylate the tyrosine-containing peptides, angiotensin II and poly(Glu,Tyr)4:1. Triton X-100 extracts from HL-60 cells treated with dimethyl sulfoxide were subjected to tyrosine-agarose chromatography, polypropyl aspartamide HPLC and HPLC using an antiphosphotyrosine IgG-derivatized column. Overall purification was 2700-fold for p98 and 1800-fold for p60. p60 and p98 are phosphorylated exclusively on tyrosine residues and can use poly(Glu,Tyr)4:1, histone H1 and vasoactive intestinal peptide as substrates. Poly(Glu,Tyr)1:1 and poly(Glu,Ala,Tyr)6:3:1 were not effective substrates for p60 and p98. The activity of p60 was dependent on Mn<sup>++</sup>, whereas p98 exhibited Mg<sup>++</sup> dependence. Both p60 and p98 were immunoprecipitated by an anti-v-src monoclonal antibody but only p98 was immunoprecipitated by an anti-v-fps/fes antibody. V<sub>8</sub> protease digestion of p60 revealed one major proteolytic fragment containing phosphotyrosine, whereas, V<sub>8</sub> protease digestion of p98 produced two major peptides that were phosphorylated on tyrosine residues. These results suggest that although p98 and p60 may possess some epitopic similarities, they have distinguishing phosphorylation sites. Moreover, p98 in contrast to p60, appears to be strictly associated with granulocytic/monocytic differentiation and related to the cellular fps/fes protooncogene.
6. We have recently identified a 98 kDa protein possessing tyrosine protein kinase activity that is highly expressed only in differentiated HL-60 cells. To obtain a better understanding of the involvement of p98 in this process, we have used proteins used as substrates by p98 to block its endogenous activity, as well as a DMSO-resistant subline of HL-60 cells. Cells treated for 4 days with DMSO or interferon- $\gamma$  differentiate to a granulocyte or monocyte phenotype, respectively, and express p98 activity. Coaddition of the p98 substrates, poly(Glu,Tyr)1:1 or



vasoactive intestinal peptide inhibited differentiation by 60% and blocked p98 activity. DMSO-resistant HL-60 cells can be sensitized to respond to DMSO by cotreatment with interferon- $\alpha$ . Under these conditions, p98 is expressed in comparison to its absence in untreated cells or in resistant cells treated only with DMSO. Thus, these results indicate that there is an obligatory association between the expression of p98 and granulocyte/monocyte differentiation, and suggests that p98 may be a receptor for factors associated with this phenomenon.

7. HL-60 cells differentiate to a macrophage-like cell in response to phorbol esters such as TPA. HL-60/ADR cells are an ADR-resistant clone which is cross-resistant to unrelated drugs and differentiating agents with the exception of TPA. On the contrary, HL-60/ADR cells are more responsive to TPA than HL-60 cells. Since PK-C is a cellular TPA receptor, we have examined the role of this enzyme in the response of HL-60 and HL-60/ADR cells to TPA. DEAE cellulose chromatography of cell extracts revealed HL-60/ADR cells contained 3-fold more PK-C HL-60 cells. After 2 day treatment with 10 nM TPA, 70-80% of the PK-C activity disappeared in both cell lines. Coincident with these changes was the appearance of vinculin phosphorylation in vitro in a  $Ca^{++}$  and phospholipid-independent manner in extracts from TPA-treated HL-60 cells which was not present in untreated cells. In contrast, untreated HL-60/ADR cells exhibited a high level of vinculin phosphorylation which was reduced 50% after TPA treatment. These results suggest that changes in PK-C are inversely related to vinculin phosphorylation after TPA treatment and that the proteolysis of PK-C to a  $Ca^{++}$  and phospholipid-independent form may be the process associated with the utilization of vinculin as an endogenous substrate.

#### Publications

1. Zylber-Katz, E., Knode, M.C. and Glazer, R.I.: Retinoic acid promotes phorbol ester-initiated macrophage differentiation in HL-60 leukemia cells without disappearance of protein kinase C. Leukemia Res. 10: 1425-1432, 1986.
2. Glazer, R.I., Chapekar, M.S., Hartman, K.D. and Knode, M.C.: Appearance of membrane-bound tyrosine kinase during differentiation of HL-60 cells by immune interferon and tumor necrosis factor. Biochem. Biophys. Res. Commun. 140: 908-915, 1986.
3. Chapekar, M.S., Hartman, K.D., Knode, M.C. and Glazer, R.I.: Synergistic effect of retinoic acid and calcium ionophore A23187 on differentiation, c-myc expression and membrane tyrosine kinase activity in human promyelocytic leukemia cell line HL-60. Mol. Pharmacol. 31: 140-145, 1987.
4. Glazer, R.I., Chapekar, M.S., Hartman, K.D., Knode, M.C. and Yu, G.: Induction of membrane-bound tyrosine kinase activity in human promyelocytic leukemia cells by differentiating agents. In Goheen, S.C. (Ed.): Membrane Proteins: Proceedings of the Membrane Protein Symposium. Richmond, CA, 1987, pp 715-728.



5. Glazer, R.I., Yu, G. and Knodel, M.C.: Analysis of tyrosine kinase activity in cell extracts using nondenaturing polyacrylamide gel electrophoresis. Anal. Biochem., in press.
6. Glazer, R.I.: Differentiation of malignant cells as a new mode of chemotherapy. In Elsebail I. (Ed): Current Treatment of Cancer. Heidelberg, UICC-Springer-Verlag, in press.
7. Glazer, R.I.: Cellular oncogenes and their gene products as potential targets for the differentiation and inhibition of cancer cells. CRC Critical Reviews in the Pharmacological Sciences, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CM 06180-02 LBC

PERIOD COVERED  
October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Sulphydryl Group in Cancer Cell Growth, Metastasis and Chemotherapy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Marco Rabinovitz Research Chemist LBC, NCI

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LAB/BRANCH  
Laboratory of Biological Chemistry

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TOTAL MAN-YEARS: 1.25	PROFESSIONAL: 1	OTHER: .25
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(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A new homocysteine derivative, S-methylthio-DL-homocysteine (SMETH) was synthesized and found to be cytotoxic to L1210 cells in culture. Its cytotoxicity was increased in the presence of leucine, methionine and several methionine analogs, at millimolar concentrations, by as yet an undetermined mechanism. Adenosine and adenylate also promoted SMETH cytotoxicity but only in the presence of deoxycorformycin. The cobalt chelates hydroxocobalamin and cobalt phthalocyanine tetrasulfonate increased cytotoxicity, possibly by maintaining an oxidative milieu which prevented the reductive degradation of SMETH. Copper ion was an especially effective synergist and permitted a threshold concentration of SMETH, 25  $\mu$ M, to become fully inhibitory.



### Objectives

The general goals of this project are directed toward an understanding of the factors influencing the sulphydryl-disulfide status of the cell and cellular milieu, and the application of this knowledge in the development of selectively toxic regimens for chemotherapy. This portion of the project is focused on an improved method for delivery of homocysteine to cells via its methylthio-derivative and introduction of mechanisms for modulating its cytotoxicity by metabolic alterations which may be applicable to chemotherapy.

These include concurrent incubation with compounds which decrease ATP levels of cells and have also been reported to have some selectivity as tumor inhibitors, e.g. rotenone, gossypol. Also under consideration is the concurrent application of amino acids which have been reported to selectively inhibit S-adenosyl-methionine synthetase (ATP: L-methionine S-adenosyltransferase) of tumor cells. Attempts will be made to decrease extracellular destruction of SMETH via sulphydryl-disulfide interchange by addition of compounds known to promote sulphydryl oxidation.

### Major Findings

A new homocysteine derivative, S-methylthio-DL-homocysteine (SMETH), was designed to promote the uptake of homocysteine via one of the aliphatic amino acid transport systems. It was cytotoxic in the micromolar range to L1210 cells in culture but promoted cell lysis only at higher concentrations (200  $\mu$ M). Attempts were therefore made to potentiate its activity.

#### A. Amino Acid Analogues

Several methionine analogs were investigated as possible potentiators of SMETH cytotoxicity in view of their ability to block S-adenosylmethionine synthesis, in many cases with tumor cell specificity (Sufrin, J.R. and Lombardini, J.B., Mol. Pharmacol. 22 752, 1982). None were better than leucine. Of particular interest was the finding that cycloleucine (1-aminocyclopentane-1-carboxylic acid, NSC 1026) was essentially an ineffective SMETH synergist although it is chemotherapeutic and a potent inhibitor of S-adenosylmethionine synthesis. Also L-methionine, at millimolar concentrations, promoted SMETH cytotoxicity, although one would expect it to effect a decrease by its stimulation of S-adenosylmethionine synthesis. These observations call into question whether SMETH cytotoxicity is due to promotion of S-adenosylhomocysteine synthesis and an increase in methylation inhibition as defined by the S-adenosylhomocysteine/S-adenosylmethionine ratio.

#### B. Adenosine and Adenylic Acid

Adenosine and adenylic acid promoted the cytotoxicity of SMETH only in the presence of deoxycoformycin to block their deamination. The effect was not marked and the potency of a threshold concentration of SMETH, 25  $\mu$ M, was not increased. Attempts to promote intracellular adenosine formation with rotenone and deoxycoformycin did not potentiate SMETH cytotoxicity. However, a schedule employing rotenone and SMETH did increase the lifespan of P388 bearing mice to a small extent.



### C. Hydroxocobalamin and Cobalt Phthalocyanine Tetrasulfonate.

Both of the above cobalt chelates have long been recognized as potent catalysts for the oxidation of sulphhydryl groups and were found to promote the activity of SMETH. This activity may be due to their ability to oxidize thiol compounds secreted by cells which would destroy SMETH before it could enter cells.

### D. Copper Ion.

A low concentration of copper ion is also a potent catalyst for oxidation of thiol compounds but it interacted with SMETH in a manner both qualitatively and quantitatively different than observed with the cobalt chelates. For example, a low concentration of copper (10  $\mu$ M) could convert a threshold concentration of SMETH (25  $\mu$ M) into a total inhibitor. This marked synergism was not seen with other ions such as Co<sup>++</sup>, Ni<sup>++</sup>, Zn<sup>++</sup>, Mn<sup>++</sup>, Cr<sup>+++</sup>, Fe<sup>++</sup>, and promoted cellular swelling not observed with the cobalt chelates. Highly cytotoxic formulations, containing SMETH, copper ion and the chelating agent, nitrilotriacetic acid were also cytotoxic to L1210 cells in culture but non-toxic to mice. Aggressive treatment with such formulations did not increase the life span of tumor bearing mice but appeared to decrease morbidity.

### Proposed Course

The unique synergistic potency of the SMETH-copper interaction makes this our principal target for further investigation. At this stage nothing is known about this interaction other than the lower homolog, S-methylthio-L-cysteine does not produce a copper dependent cytotoxicity.

1. To determine whether the SMETH-copper cytotoxicity is purely chemical or biochemical in nature, SMETH prepared from the unnatural D-homocysteine will be compared with the L-form for copper dependent cytotoxicity. Since the D-form of homocysteine does not participate in enzymatic reactions a decision can be made on which course of investigation to follow.
2. Drug and copper will be obtained in a labeled form to see if cellular uptake is stoichiometric and due to a SMETH-copper complex.
3. Characteristics of the SMETH-copper interaction, both in vitro and in vivo, will be evaluated.

### Publications

1. Rabinovitz, M.: Emerging Evidence for Control of Monovalent Cation Homeostasis as a Critical Target in Alkylating Agent Resistance. In Kessel, D. (Ed.): Resistance to Antineoplastic Drugs. Boca Raton, CRC Press, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CM 06181-02 LBC

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interaction of GTP-Binding Membrane Proteins with Cellular Components

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Richard A. Kahn Senior Staff Fellow LBC, NCI

Others: Janet Holden Visiting Fellow LBC, NCI

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 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Activation of the ras oncogene has been implicated as the causative agent in as many as 30% of all human tumors. Yet in spite of extensive work on the ras gene, including the identification of at least seven different ras genes and an increasing number of ras-like genes, almost nothing is known about the function of the ras proteins in higher eukaryotes. A recently characterized protein, termed ARF, is a component of the adenylyl cyclase system and shares several features with ras, including size, location, and ability to bind GTP. Characterization of the binding and hydrolysis of guanine nucleotides by ras and ARF will be undertaken. A systematic search will then be conducted to identify factor(s) which increase either the exchange or hydrolysis of guanine nucleotides by these regulatory proteins. These studies should locate cellular targets for these proteins and may identify the physiological role of these proteins in cellular metabolism or proliferation.



Research on the regulation of the adenylate cyclase system has led to the discovery of a family of homologous GTP-binding, regulatory proteins (G-proteins). Five distinct members have been identified and purified, though only two have defined roles in the regulation of adenylate cyclase activity. Although implicated as regulators of  $K^+$ ,  $Ca^{++}$  and other ion channels, phosphoinositide metabolism, and cellular proliferation, the roles of the other G-proteins are not yet known.

A number of recent techniques in molecular biology have led to the discovery of another family of GTP-binding membrane proteins which are products of the *ras* oncogene. Seven distinct *ras* genes have been identified. Ras proteins have been shown to activate adenylate cyclase from one strain of yeast but have no known function or role in any higher eukaryotes.

A novel G-protein has been purified and characterized by the PI. This protein, termed ARF, interacts with the adenylate cyclase system and shares many of the GTP-binding characteristics of ras. Both proteins have a molecular mass of 21,000 daltons.

### Objectives

In all cases where a cellular role is known the activity of a guanine nucleotide binding protein is controlled by the binding of GTP. Hydrolysis of the bound GTP results in deactivation. Thus, knowledge of the factors which control the binding and hydrolysis of GTP will also identify elements upstream and downstream of the regulatory proteins. The objective of this work will be to utilize the nucleotide binding properties of these two proteins, ras and ARF, to identify cellular targets and pathways controlled by either of these proteins.

### Methods employed

The principal method utilized will be radioligand binding studies of nucleotide binding to purified ARF and ras proteins. Factors which increase either the off-rate of bound GDP or hydrolysis of bound GTP will be screened for in tissue extracts. Standard protein purification methods will be utilized to further characterize any activity present.

### Major findings

The half-time of GDP release from ARF is 24 min under certain conditions. This can be increased more than 10-fold ( $t_{1/2} = 2$  min) by the addition of a detergent extract of bovine brain membranes. The factor(s) responsible for this change in affinity of ARF for GDP is under investigation.  $Mg^{++}$  and lipid have recently been found to have much greater effects on the kinetics and steady-state levels of nucleotide binding to ARF. The roles of these factors on protein conformation and nucleotide binding will be explored in detail.



Publications

1. Kahn, R.A. and Gilman, A.G. The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP-bindign protein. J. Biol. Chem., 261: 7906-7911, 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06182-02 LBC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Genetic &amp; Immunologic Analyses of a Novel GTP-Binding Regulatory Membrane Protein

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Richard A. Kahn Senior Staff Fellow LBC, NCI

Others: Jenny Sewell Microbiologist LBC, NCI

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(a1) Minors

(a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

ARF is a recently characterized GTP-binding regulatory membrane protein component of the adenylate cyclase system. These studies are aimed at the elucidation of the role of ARF in signal transduction, differentiation, and proliferation. In the past year, the bovine ARF gene has been cloned and sequenced, and monospecific peptide antibodies have been raised to the GTP binding domain in the protein. DNA and antibody probes have been used to detect the gene and protein in yeast cells. These probes will be used to clone the yeast ARF gene, which will then be sequenced and used in gene disruption and gene replacement studies. The yeast system will also be used to study the expression of normal and mutated ARF genes. Corollary studies will be performed with the antibody probes on the cellular localization and functions of the expressed proteins.



ARF has recently been identified as a 21,000 Da GTP-binding regulatory membrane protein. Activated ARF binds to the stimulatory, regulatory component (Gs) of adenylate cyclase and allows cholera toxin to irreversibly activate the cyclase. Though apparently a component of the adenylate cyclase complex, the physiological role of ARF is unknown. ARF is present in the plasma membrane of every eukaryotic tissue or cell type examined. Attempts to isolate ARF-deficient mutant cell lines have been unsuccessful in a number of different laboratories. ARF has the same molecular mass and shares the GTP binding characteristics with products of the ras oncogene. There are also clear differences between these proteins, e.g. no immunological cross-reactivity. Ras proteins have been implicated in changes in cyclic nucleotide levels in several tissues and have recently been shown to directly stimulate adenylate cyclase in yeast. The role of ras proteins in adenylate cyclase of higher eukaryotes is unknown but is clearly not a direct coupling to the cyclase.

#### Objective

The objective of this work is the cloning and sequencing of yeast ARF. Subsequent work will focus on defining the role of yeast ARF in cell proliferation and physiology. In addition, monospecific peptide and protein antibodies to ARF will be used to further characterize the cellular location and cellular requirement for ARF.

#### Methods Employed

The methods employed will include the screening of a genomic yeast plasmid library with bovine ARF cDNA probes to obtain a full length DNA coding for the yeast ARF gene followed by the nucleotide sequencing of the DNA and comparison to other known sequences. The yeast ARF gene will be used to perform gene deletion experiments to determine if ARF is necessary for cell survival. The yeast system will also be used as a means of expressing other ARF genes in a eukaryotic cell under normal regulatory influence, and later, to determine the effects of mutagenesis on gene function.

#### Major Findings.

Cloning and sequencing of the ARF gene from bovine adrenal cells and comparison to the sequences of other GTP-binding proteins has demonstrated that ARF contains homology to both the ras and the trimeric G-protein families. While all are proposed to be members of a superfamily of GTP-binding regulatory proteins, ARF may represent an evolutionary intermediate between the two families. Data suggest that more than one ARF gene may exist. Cellular localization of ARF by immunoblotting has, surprisingly, revealed that ARF is predominantly a cytosolic protein. The roles of post-translational acylation and cellular localization are becoming increasingly important in defining cellular function and targets.

#### Proposed Course

1. Clone yeast ARF and compare the primary sequence to bovine ARF, ras and other GTP-binding proteins. Delete the endogenous gene and determine phenotype.



Replace the endogenous gene with the bovine gene or mutant forms of an ARF gene. Transform E.coli to get high level expression of ARF protein for use in other studies.

2. Further characterize the high titer monospecific antibodies to ARF. Determine if any alter ARF function when bound. Microinject inactivating ARF antibodies and/or altered ARF constructs into normal cells. Perform immunocytochemistry to determine cellular location of normal and mutant ARF proteins.

Publications

1. Kahn, R.A. and Gilman, A.G. The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP-binding protein. J. Biol. Chem., 261: 7906-7911, 1986.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06187-01 LBC

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inhibitors of Protein Kinases as Potential Chemotherapeutic Agents for AIDS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Robert I. Glazer	Pharmacologist	LBC, NCI
Others:	Angelo Aquino	Visiting Fellow	LBC, NCI
	Gang Yu	Visiting Fellow	LBC, NCI
	Carlo-Fredrico Perno	Visiting Fellow	MB, NCI
	Candace Pert	Pharmacologist	CNB, NIMH
	Victor E. Marquez	Visiting Scientist	LMC, NCI

COOPERATING UNITS (if any)

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Clinical Neuroscience Branch, NIMH  
Laboratory of Medicinal Chemistry, DCT, NCI

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 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The aims of this proposal are to: 1) study the phosphorylation of the HTLV-III antigen, p24 by T cell tyrosine kinase and protein kinase C and 2) test and design inhibitors of these phosphorylation reactions as potential inhibitors of HTLV-III replication or infectivity. It is hoped that inhibitors of p24 phosphorylation will yield a new class of HTLV-III-specific inhibitors unrelated to the agents such as azidothymidine currently being employed, and thus offer a second line of therapy for AIDS.



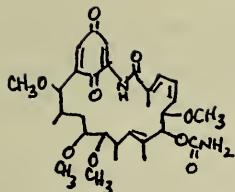
There is a close association between HTLV-III infection and AIDS as determined by serological evidence of antibodies to HTLV-III antigens in patients with AIDS. In seropositive patients, there is a high serum titer to HTLV-III proteins of 120, 66, 51, 41, 31, 24 and 17 kD. Among these peptides, the p120 and p41 are glycoproteins associated with the viral envelope and p24 is the major viral core (gag) protein. Metabolic labeling with  $^{32}\text{P}$  of cells infected with HTLV-III results in one major phosphopeptide, pp24. There are three phosphorylation sites in pp24, ArgGlySer, ArgMetTyr and ArgPheTyr in the middle of the peptide toward the COOH terminus. Whether the phosphorylation of pp24 is accomplished by constitutive protein kinases of the infected helper T-cell or by a virus-associated protein kinase is uncertain.

There is precedent for the importance of retroviral tyrosine kinase activity in the ability of the virus to transform cells. It is known that transformation by the Rous sarcoma virus is dependent on a functionally competent pp60<sup>V-SRC</sup> tyrosine kinase. The transforming gene product, pp60<sup>V-SRC</sup> phosphorylates host cellular proteins on tyrosine as well as itself with the sequence LeuIleGluAspAsnGluTyr(P)ThrAlaArg associated with the autophosphorylation site in pp60<sup>V-SRC</sup>. Another example of the importance of cellular phosphorylation reactions in the function of viruses is the stimulation by protein kinase C (Ca<sup>++</sup> and phospholipid-dependent protein kinase) of the association of polyoma virus middle T antigen with the cellular homolog of pp60<sup>V-SRC</sup>, pp60<sup>C-SRC</sup>. Phosphorylation of this transforming viral protein by two protein kinases, the first to increase its association with a tyrosine kinase and the second to increase its content of phosphotyrosine, may be an important clue to the transforming ability or replicative capacity of retroviruses. Tyrosine phosphorylation may also play a role in autoimmune diseases. Mice developing this trait have shown unregulated tyrosine phosphorylation on a component of the T-cell receptor in comparison to normal T-cells.

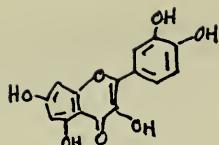
Thus, it is plausible that host cell phosphorylation reactions play a crucial role in the ability of the retrovirus to replicate and destroy the cell. It remains a mystery as to whether host cell protein kinase C is required for HTLV-III replication, whether serine phosphorylation is required for or enhances the tyrosine phosphorylation of the viral protein, whether the tyrosine kinases in T-cells show greater specificity for HTLV-III proteins than other cellular tyrosine kinases, or if interruption of viral protein phosphorylation on Ser and/or Tyr interferes with viral replication.

Of obvious import to these questions will be the design of pharmacological agents with the ability to specifically inhibit protein kinase C and tyrosine kinase activities. Three compounds which may serve as prototypes for inhibitors of tyrosine kinases are herbimycin A and quercetin, both inhibitors of pp60<sup>V-SRC</sup> tyrosine kinase, and erbstatin, an inhibitor of epidermal growth factor (EGF) tyrosine kinase. Herbimycin A is a benzoquinoid ansamycin antibiotic with antiviral and antitumor activity. Quercetin, is a plant bioflavonoid with moderate inhibitory activity against pp60<sup>SRC</sup> and protein kinase C. Erbstatin is an isoflavone compound isolated from a soil fungus and inhibits EGF receptor tyrosine kinase directly.

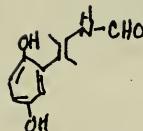




herbimycin A



quercetin

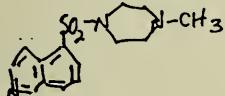


erbstatin

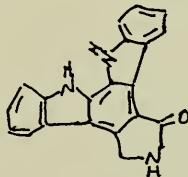
## Structures of inhibitors of tyrosine kinases

There appears to be enough structural similarity between these agents to be able to model analogs with potential inhibitory properties against tyrosine kinase. For example, they all possess a phenolic or quinone ring with a flavone-like structure. Structure-activity relationships among these classes of inhibitors will be explored with commercially-available compounds and further drug design will be conducted in collaboration with Dr. Victor Marquez, LMC, NCI.

There are two classes of protein kinase C inhibitors which are noteworthy. Hidaka's lab synthesized a series of isoquinoline sulfonamides, the most potent of which is H-7.



H-7



staurosporine C

H-7 has been shown to inhibit protein kinase C-dependent phosphorylation in platelets and HL-60 leukemia cells *in vitro*, and thus may be a good initial candidate to test against HTLV-III-infected cells. Staurosporine is an indole-carbazole microbial alkaloid and is extremely inhibitory against protein kinase C ( $IC_{50} \sim 3nM$ ) as well as being highly cytotoxic to HeLa cells. Thus, analogs of H-7 or staurosporine may also find utility as inhibitors of virus-associated phosphorylation reactions.

Since HTLV-III-associated p24 contains one phosphoserine and two phosphotyrosine residues, and since there appear to be tissue-specific tyrosine kinases as well as a tissue-specific distribution of protein kinase C, it would appear reasonable to test inhibitors of tyrosine kinase and protein kinase C as inhibitors of HTLV-III infectivity *in vitro*. Since HTLV-III specifically infects helper T-cells, the T-cell tyrosine kinase, p58, which is found only in T lymphocytes and not B lymphocytes and is related to the p58 found in human T cell leukemias and in the human T-cell lymphoma LSTRA will be isolated. In addition, purification of protein kinase C from T-cells or other tissue sources would serve as a comparison for the specificity of the compounds tested as inhibitors of serine and/or tyrosine phosphokinases. The substrate will be the



p24 HTLV-III antigen and the active phosphorylation site of 8-10 amino acids will later be synthesized as a synthetic substrate for the various kinases reactions. Those compounds showing inhibitory activity will be tested in an in vitro HTLV-III screening system.

In studies of the differentiation-associated tyrosine protein kinase or p98, we noticed the vasoactive intestinal peptide (VIP) possessed a peptide sequence similar to that of the autophosphorylation site of p60<sup>v</sup>-SRC tyrosine protein kinase. Indeed, VIP was a very good substrate for p98. Studies by Pert et al. have shown that a pentapeptide sequence in the N-terminus of VIP is conserved in the gp120 viral envelope protein of HTLV-III, and that this peptide, termed peptide T, can block the infectivity of HTLV-III in vitro. Thus, we are examining whether peptide T can serve as a substrate or can influence the activity of p98 and other tyrosine protein kinases, and whether the therapeutic effectiveness of peptide T is associated with tyrosine protein kinases or perhaps serine/threonine kinases.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CM 06188-01 LBC

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mutagenesis Studies to Map Regulatory Protein Domains

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

ARF, ras(p21), src, cAMP-dependent protein kinase, and the family of G-proteins, are all regulatory proteins whose cellular localization and/or activities are affected by covalent attachment of the fatty acids, myristate or palmitate. The role of post-translational covalent acylation of regulatory proteins will be examined by generating a series of mutant genes using site-directed mutagenesis. The normal and mutant cDNAs will then be expressed and proteins purified for tests of intrinsic enzymatic activities as well as the ability to participate in regulatory, protein-protein interactions. Immunocytochemistry will be used to determine the cellular localization of mutant proteins expressed in appropriate cellular systems. In addition, the mutant proteins will be tested as potential substrates or inhibitors of myristyl and palmityl transferases.



A superfamily of GTP binding regulatory proteins has been defined by the characterization and sequencing of the ras, trimeric G-protein, and monomeric GTP-binding protein families. Activation and deactivation of members of this superfamily are controlled by the regulated binding and hydrolysis, respectively, of GTP. These proteins have regulatory roles in cell proliferation, differentiation, and signal transduction. In those cases where studies have been performed, the cellular localization of the GTP-binding protein has been shown to be of critical importance. The ras proteins have been shown to be palmitoylated, resulting in attachment to the inner leaflet of the plasmalemma. The G-protein  $\alpha$  subunits have been suggested as possible sites for myristylation. Proof of this modification and demonstration of any direct role in membrane localization is lacking, however.

The techniques of molecular biology currently allow the construction of genes with specific alterations in the coding region of any cloned protein. These techniques will be employed to study the nucleotide binding sites and acylation acceptor sites on members of the GTP binding regulatory protein superfamily.

#### Objectives

We will construct mutant genes with specific changes in the coding region of specific proteins. We will initially make mutants of ARF, the 21 kDa GTP-binding component of adenylate cyclase, as it has both GTP-binding domains and regions for acylation which may dramatically alter cellular localization. ARF is also one of the few GTP-binding proteins possessing assayable activity, as well as DNA, RNA, and antibody probes. The mutant genes will then be expressed in either *E.coli* or yeast to generate altered proteins for biochemical and immunohistochemical analysis. The effects of modifications of specific amino acids or regions of the protein will be assessed with regard to functional consequences.

#### Methods employed

Site-directed mutagenesis will be employed to construct the mutant genes for this investigation. The genes themselves have been cloned and sequenced in this laboratory or are currently available from other laboratories. Mutants will then be transfected into *E. coli* for expression of protein. Radionucleotide binding studies, biochemical assays, and Western blotting will all be performed in the laboratory using methods already in use.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06189-01 LBC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Stable Isotope Studies of Glutamine Requirements for Tumor Cell Growth

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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D.W. Zaharevitz Staff Fellow LBC, NCI  
R.L. Cysyk Pharmacologist LBC, NCI

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2

1.5

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(a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies were initiated to evaluate the role of glutamine as a respiratory fuel and to determine the fate of its carbon and nitrogen atoms for intermediary metabolism necessary for tumor growth. To facilitate this investigation, automated GC/MS methods were developed to simultaneously quantify and measure the isotopic content in amino acids, various sugars, lipids, cholesterol, and TCA intermediates. Preliminary in vitro studies with L1210 ascites tumors grown in mice were conducted to develop the approach to be used for in vitro studies with human tumor lines and in tumors and normal tissue in vivo. Incubation of L1210 tumors with various isotopic variants of glutamine and uniformly labeled glucose resulted in the following observations. (1) Large amounts of glucose and glutamine were consumed averaging 7.5  $\mu$ moles/min-g dry weight and 2.2  $\mu$ moles/min-g dry weight, respectively. (2) 80-90% of [1,2,3,4,5,6-13C]glucose used was metabolised to [1,2,3-13C]lactate. (3) 89% of the 5-amido nitrogen was recovered as [15N]H3 and 80% of the alpha nitrogen underwent transamination to form [15N]alanine. Small amounts of the glutamine alpha 15N were recovered in glutamate, aspartate, proline, serine, and pyroglutamate. (4) Preliminary data indicate that glutamine transaminase activity may be present in L1210 tumors resulting in an alternate pathway to alpha-ketoglutarate via transamination to alpha-ketoglutaramate and omega amidase hydrolysis. (5) Incubation of L1210 tumors with [2,3,3,4,4-[2H]-glutamine provided evidence of a truncated TCA cycle, with glutamine supplying most of the carbons for the TCA cycle and cytosolic pyruvate supplying minimal acetylCoA for oxidation in the TCA cycle. Most of the citrate formed in the mitochondria was probably exported to the cytosol.



Intermediary metabolism of tumor cells is different from normal proliferating cells in the following manner: enhanced aerobic glycolysis and glucose uptake, enhanced glutaminolysis, enhanced nucleic acid synthetic capacity, enhanced lipid synthesis, reduced pyruvate and acetylCoA oxidation rate, lower sensitivity to oxygen and a lower hormone requirement. Numerous postulates have been advanced concerning the role of carbohydrate and glutamine metabolism (these two substrates represent the major nutrients used) in cell proliferation and tumor formation. In order to establish the interrelationship between the glycolytic and glutaminolysis pathways and their regulation, it is necessary to measure the true activities of these pathways in the intact tumor cell and in tumors and normal tissue in the intact animal.

The use of stable labeled compounds as tracers combined with mass spectrometric techniques in some cases are more advantageous for these type of studies. These advantages have been discussed under project Z01 CM 06165-02 LBC in this report. Multiple label studies using  $^{15}\text{N}$ ,  $^{2}\text{H}$ , or  $^{13}\text{C}$  labeled glutamine in combination with  $^{13}\text{C}$  or  $^{2}\text{H}$  labeled glucose should allow us to address some of the questions regarding the importance of glutaminolysis and aerobic glycolysis as required for tumor cell growth.

### Objective

The objective of this project is two-fold: (1) To establish the metabolic fate of glutamine nitrogen and its carbon backbone in various human tumor cell lines with respect to respiration and anabolic reaction necessary for cell growth and (2) to develop stable-label methodology and models to study flux through the glycolytic and glutaminolysis pathways *in vivo* in tumors and normal tissue.

### Measurement of glutamine disposition in L1210 tumors in vitro.

Automated GC/MS methods were developed for studying the fate of glutamine nitrogen and carbon atoms and glucose carbon atoms in tumor cells using stable isotope methodology. The methods developed allows the simultaneous quantification and isotopic enrichment measurements in most amino acids, glucose, lactate, malate, citrate, fatty acids, and cholesterol. Samples from incubations, tissue extracts and plasma have been analysed.

Preliminary glucose and glutamine disposition studies using stable labeled isotopes were initiated in L1210 cells grown in mice. L1210 cells ( $5-8 \times 10^6$  cells/ml) in MEM fortified with varying concentrations of glucose, glutamine and pyruvate were incubated for 120 min and samples were obtained at 0 min, 30 min and 120 min for analysis. In order to determine the fate of the glucose and glutamine in the tumors various isotopic variants of these substrates were employed. Using  $[1,2,3,4,5,6-13\text{C}]$ glucose and either  $[2-15\text{N}]$ glutamine or  $5-15\text{N}-7.5$  moles/min-g dry weight and 2.2 moles/min-g dry weight, respectively for glucose and glutamine. The rates were linear with time over 120 min. 90% of the added glucose was recovered as  $[1,2,3-13\text{C}]$ lactate. Following incubation with  $[5-15\text{N}]$ glutamine 89% of the amide nitrogen metabolized was recovered as  $[15\text{N}]$ H<sub>2</sub>O. The alpha nitrogen of glutamine was determined after incubation with  $[2-15\text{N}]$ glutamine. The alpha nitrogen utilized was recovered in the following compounds: alanine (61%), glutamate (16%), aspartate (1%) and the remainder in serine, threonine, and pyroglutamate. Incubation with  $[2,3,3,4,4-2\text{H}]$ glutamine



and the resulting distribution in TCA intermediates were consistent with the following: (1) a truncated TCA cycle with very little oxidation of citrate to alpha ketoglutarate, (2) less than 15% cytosolic pyruvate derived from glucose or the media oxidized by the TCA cycle and (3) mitochondrial malic enzyme and malate dehydrogenase activity providing oxaloacetate and pyruvate used for oxidation to acetylCoA and production of citrate.

Stable isotope methods for measuring flux of intermediates in vivo.

In order to establish the use of glutamine nitrogen in tumors and normal tissue in vivo and its relationship to the glycolysis pathway important fluxes to be measured include: (1) pyruvate and lactate to alanine, (2) dihydroxyacetone to aspartate and (3) dihydroxyacetone to serine. To evaluate the possibility of establishing the rate of alanine rate of production from pyruvate, L1210 tumors were incubated with  $[2-^{15}\text{N}]$ glutamine and  $[1,2,3,4,5,6-^{13}\text{C}]$ glucose. GC/MS analysis of the alanine formed and application of the probability theory and F calculations used previously for pyrimidine rates of de novo synthesis showed that the alanine pool measured after 120 min was entirely derived from the lactate pool. These preliminary experiments demonstrate the feasibility of separating the alanine or aspartate formed from glycolysis in the tumor cells as opposed to that obtained from plasma in the intact animal.



## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Molecular and Cellular Pharmacology of Nucleoside Analogs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others: Kathleen D. Hartman Chemist LBC, NCI

Marian C. Knode Biologist LBC, NCI

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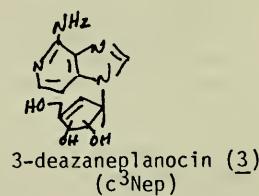
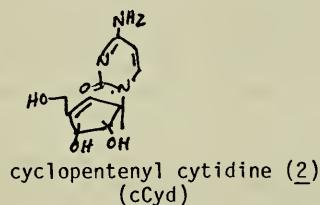
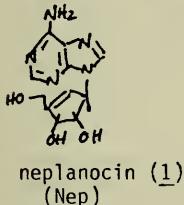
(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanism of action of carbocyclic nucleoside analogs was investigated in human carcinoma cell line HT-29 and human promyelocytic leukemia cell line HL-60. The 3-deaza analog of neplanocin, c3Nep, was as effective as an inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase as neplanocin but possessed 1/10 the cytotoxicity, did not effectively inhibit RNA methylation and was not metabolized to an AdoMet-like metabolite. c3Nep was ineffective as a differentiating agent in HL-60 cells. The cyclopentenyl cytidine analog (cCyd) possessed a potent inhibitory effect on CTP synthesis and subsequently, DNA synthesis with moderate cytotoxicity against HT-29 and HL-60 cells. However, this agent proved to be a very effective differentiating agent for HL-60 cells, probably as a result of its S phase specific effects at noncytotoxic concentrations. In other studies, the adenosine deaminase inhibitor, 2'-deoxycoformycin (dCF), was found to produce complete remissions in hairy cell leukemia in phase II clinical trials.



Molecular and Cellular Pharmacology of Nucleoside Analogs. Studies over the past year have involved our continuing collaboration with the Laboratory of Medicinal Chemistry, on the mechanism of action of carbocyclic nucleoside analogs. The parent analog, cyclopentenyl adenosine or neplanocin (1) proved to be an effective inhibitor of RNA methylation in human colon carcinoma cell line HT-29 (Fig. 1). Subsequent studies on the effect of neplanocin on the differentiation of human promyelocytic leukemia cell line HL-60 confirmed this activity and indicated that reduced methylation of RNA and DNA as well as the expression of the protooncogene, *c-myc*, did not lead to complete maturation of this leukemia. In contrast, the cyclopentenyl cytidine analog (2) produced rapid and complete differentiation of HL-60 cells to the granulocytic phenotype with less cytotoxicity than neplanocin. Accompanying these changes was a pronounced inhibition of CTP synthesis and subsequently, DNA synthesis, and a rapid reduction in *c-myc* RNA expression. The relationship between methylation and differentiation was further documented in our recent studies of 3-deaza-neplanocin (*c*<sup>3</sup>Nep) (3), an inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase (Fig. 1). *c*<sup>3</sup>Nep has proven to be the most potent inhibitor of AdoHcy hydrolase to date and elevates intracellular levels of AdoHcy in HT-29 or HL-60 cells to an amount comparable to that of neplanocin which is also an inhibitor of the hydrolase. However, unlike neplanocin, *c*<sup>3</sup>Nep is neither markedly cytotoxic, inhibitory to rRNA methylation, nor is metabolized like neplanocin to the nucleotide or S-adenosylmethionine-like metabolite. Thus, *c*<sup>3</sup>Nep and Nep have allowed us to dissect the influence of inhibition of



AdoHcy hydrolase on RNA methylation, cytotoxicity and differentiation by their diverse and specific pharmacological activities.

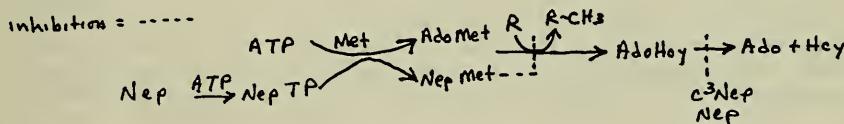


Fig. 1. Sites of action of neplanocin (Nep) and 3-deazaneplanocin (*c*<sup>3</sup>Nep)

*c*<sup>3</sup>Nep, although not an effective antiproliferative agent, has proven active against RNA viruses such as polio, coxsackie and vaccinia in preliminary screening.

In other studies of the antitumor activity of the adenosine deaminase inhibitor, 2'-deoxycoformycin (dCF), we ascertained that this agent was highly effective against hairy cell leukemia in phase II clinical trials. In contrast, patients with chronic lymphocytic leukemia only partially responded and their clinical response was unrelated to changes in adenosine deaminase activity,



levels in adenine nucleotides, or DNA breakage. Due to the change in emphasis of the research program of this laboratory, further studies of nucleoside analogs, of which this Section has engaged in for the past 10 years, will not be forthcoming.

#### Major Findings

1. The effects of the cyclopentenyl (cCyd) and cyclopentyl (carbodine) analogues of cytidine on differentiation, and nucleic acid and nucleotide biosynthesis were examined in human promyelocytic leukemia cell line HL-60. Continuous exposure for 5 days to  $10^{-8}$  to  $10^{-5}$  M cCyd or  $10^{-6}$  to  $10^{-5}$  M carbodine produced progressive inhibition of cell growth. During this exposure interval, pronounced differentiation to mature myeloid cells occurred wherein 95% of the cell population reduced nitroblue tetrazolium four days after exposure to  $10^{-7}$  M cCyd or  $10^{-5}$  M carbodine. Preceding differentiation was the inhibition of DNA synthesis which reached 10% of control levels 24 hr after exposure to  $10^{-7}$  M cCyd or  $10^{-5}$  M carbodine, while RNA synthesis was inhibited to a lesser extent. The induction of mature myeloid cells by cCyd was preceded by the pronounced inhibition of c-myc mRNA levels which was more pronounced than the reduction in total cellular RNA synthesis. During the interval of cCyd treatment, there was a rapid and striking decrease in the level of CTP, but not of UTP, ATP or GTP, where the half-life for the reduction in CTP was 1.5 to 2 hr. Following drug removal, cells treated with cCyd showed a sustained reduction in CTP levels, whereas cells treated with carbodine showed almost complete recovery of CTP levels within 48 hr. These results indicate that the reduction in CTP levels leads to rapid inhibition of DNA synthesis and reduction in c-myc mRNA levels which precede the appearance of differentiated cells.
2. 3-Deazaneplanocin ( $c^3$ Nep) was tested as an antiproliferative agent against human colon carcinoma cell line HT-29.  $c^3$ Nep produced a 16 and 34% reduction in cell viability after 24 h of drug exposure at 10 and 100  $\mu$ M concentration, respectively. Neplanocin at identical concentrations decreased viability by 31 and 87%.  $c^3$ Nep inhibited AdoHcy hydrolase in vivo as assessed by elevation in AdoHcy levels, and was found to be as effective as neplanocin as an inhibitor of AdoHcy synthesis. However, unlike neplanocin,  $c^3$ Nep neither markedly inhibited RNA methylation nor was metabolized to an S-adenosylmethionine-like metabolite. This result suggests that inhibition of AdoHcy hydrolase, while a good antiviral target, is not adequate to produce a tumoricidal response.
3. Eight patients with hairy-cell leukemia (HCL) complicated by pancytopenia, were treated with low dose regimens of the adenosine deaminase inhibitor dCF. All patients had significant hematological and clinical improvement. One patient who had been splenectomized and 5 patients with mild to moderate splenomegaly achieved normal blood counts within two months, which have been maintained for up to 18 months. Complete remissions occurred in two patients and four patients had 50-95% marrow clearance of hairy-cells. The initial dCF treatments produced a 1-3 gm/dl fall in the hemoglobin levels and one patient had a temporary reduction in granulocyte and platelet counts. Five patients had nausea/vomiting, and/or lethargy



following dCF, but there was no correlation between the plasma levels of deoxyadenosine and adenosine and the incidence or severity of these side effects. An increased incidence of infection and drug hypersensitivity may reflect the effects of dCF on the immune system. Low dose dCF is a highly effective agent in HCL.

4. Four patients with refractory chronic lymphocytic leukemia were treated with the adenosine deaminase inhibitor, 2'-deoxycoformycin (dCF), and initially received 4 mg/m<sup>2</sup> i.v. weekly. Clinical responses to therapy varied: Patient A had a minimal response, whereas Patient D showed an 85% decrease in lymphocyte count at 2 wk, and Patients B and C had intermediate responses. The pretreatment mononuclear cell adenosine deaminase activities, which ranged from 1.6 to 44.6 nmol adenosine/h/10<sup>6</sup> cells, decreased to approximately 1 nmol adenosine/h/10<sup>6</sup> cells 24 h following dCF, and increased to 15 to 50% of the pretreatment activity prior to the second drug treatment. The clinical response to dCF was unrelated to the pre- or post-treatment adenosine deaminase activities or to the rate of return of enzyme activities following treatment. The plasma deoxyadenosine levels and the leukemic cell dATP concentrations rose slightly with therapy, but there was no correlation between the magnitude of increase and clinical response. No significant levels of DNA strand breaks were observed in the leukemic cells following treatment, although the NAD levels decreased slightly in two patients. When peripheral mononuclear cells from the patients and two controls were incubated *in vitro* for 24 h with dCF and increasing concentrations of deoxyadenosine, a concentration-dependent increase in dATP and decrease in NAD were observed in both the patients and normals. The normal cells, and cells from two patients, developed a significant number of DNA strand breaks. However, there was no relationship between the formation of DNA breaks and the degree of accumulation of dATP or depletion of NAD, or between any of these changes and subsequent clinical responses to dCF. Based on this study, it appears that the anti-tumor activity of dCF in chronic lymphocytic leukemia is unrelated to the induction of DNA strand breaks or to changes in the levels of dATP or NAD in the leukemic cells.

#### Publications

1. Glazer, R.I., Cohen, M.B., Hartman, K.D., Knode, M.C., Lim, M.-I. and Marquez, V.E.: Induction of differentiation in human promyelocytic leukemia cell line HL-60 by the CTP synthetase inhibitor, cyclopentenyl cytidine analogue. Biochem. Pharmacol. 35: 1841-1848, 1986.
2. Johnston, J.B., Glazer, R.I., Pugh, L. and Israels, L.G.: The effectiveness of 2'-deoxycoformycin in the treatment of hairy cell leukemia. Brit. J. Haematol. 63: 525-534, 1986.
3. Johnston, J.B., Begleiter, A. and Glazer, R.I.: Effects of 3'-(4-morpholinyl)-3'-deaminodaunorubicin, 3'-(4-methoxy-1-piperidinyl)-3'-deaminodaunorubicin and daunorubicin on nuclear and polysomal RNA synthesis in human colon carcinoma cells *in vitro*. Cancer Lett. 33: 225-233, 1986.



4. Glazer, R.I., Knodel, M.C., Tseng, C.K.H., Haines, D.R. and Marquez, V.E.: 3-Deazaneplanocin: a new inhibitor of S-adenosylhomocysteine hydrolase and its effects in human colon carcinoma cells. Biochem. Pharmacol. 35: 4523-4527, 1986.
5. Begleiter, A., Glazer, R.I., Israels, L.G., Pugh, L. and Johnston, J.B.: Induction of DNA strand breaks in chronic lymphocytic leukemia following treatment with 2'-deoxycoformycin in vivo and in vitro. Cancer Res. 47: 2498-2503, 1987.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CM 07156-04 LBC

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October 1, 1986 to September 30, 1987

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Differentiation of Human Leukemia Cells

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Others: Yuya Abe Visiting Fellow LBC, NCI  
Noriko Takahashi Visiting Fellow LBC, NCI

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 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recently there has been interest in the possibility that "differentiation-inducers" will have utility in the treatment of some malignancies. The human promyelocytic cell line HL60 has been a useful model system in these studies. This cell line is induced to differentiate along the granulocyte pathway by retinoic acid (RA), DMSO, and HMBA and along the monocytic/macrophage pathway by vitamin D3 and TPA. More recently we have found that HL60/MRI, a cell line established from a transplantable HL60 tumor in nude mice, differentiates to monocytoid cells in response to RA and to granulocytic cells in response to DMSO and HMBA. This, as well as other differences between HL60 and HL60/MRI, prompted an investigation of the expression of the proto-oncogenes myc, fms, fos, and N-ras in these two cell lines during differentiation. Compared to HL60, the proto-oncogene myc in HL60/MRI is amplified approximately 60% less in genomic DNA and is expressed at a high transcriptional level similar to that in HL60. During differentiation there is a rapid decrease in the levels of c-myc mRNA in both HL60 and HL60/MRI. The protooncogene fms is closely related or identical to the receptor for macrophage colony stimulating factor (CSF-1). Transcripts of c-fms were detected during RA-induced granulocytic differentiation of HL60, but they did not increase in proportion to the extent of differentiation. However, in HL60/MRI, c-fms mRNA was detectable at 12 hr after treatment with RA and increased in amount as the extent of differentiation increased. These results indicate that c-fms is associated with monocytic differentiation and provide some support for a role of CSF-1 in the expression of functional parameters of mature myelomonocytic cells in addition to its role in growth of primitive cells. The expression of c-fos mRNA was observed during either RA- or DMSO-induced differentiation in serum-free medium. c-fos transcripts were not observed in the presence of serum. No major changes in N-ras expression was observed during differentiation.



### Objectives

This project seeks an understanding of the process of terminal differentiation of human myeloid cells. It is now generally agreed that some leukemias, as well as other malignancies, are diseases resulting from a block in terminal differentiation. This view suggests that viable treatment may be possible with agents that induce differentiation. To aid in this search, studies are conducted to: a) better understand the mechanism(s) of terminal differentiation; b) study the metabolism of known inducers of differentiation, e.g., RA, to aid in the development of more potent inducers; c) screen known and newly synthesized compounds for their differentiation inducing activity; and d) employ an animal model system (transplantable HL60 carried in athymic nude mice) to determine if inducers of differentiation in vitro are active by a similar mechanism in vivo.

### Methods Employed

The principal methods employed involve measurement of differentiation of human leukemia cell lines in cell culture. Most studies are conducted with either the HL60 promyelocytic cell line or with the HL60/MRI transplantable tumor cell line. Differentiation is assessed primarily by morphology and the ability of cells to reduce nitroblue tetrazolium to a formazan after treatment of cells with an inducer. Measurement of proto-oncogene expression is by immunological techniques using flow cytometry and Western blotting.

### Major Findings

1. In comparison to HL60, the proto-oncogene myc of HL60/MRI is amplified approximately 60% less in genomic DNA. There is a corresponding decrease in the expression of c-myc mRNA in HL60/MRI compared to HL60.
2. During the induction of differentiation by RA the rate of differentiation of HL60/MRI is greater than that of HL60. However, the rate of decrease (half-life) of c-myc mRNA is approximately the same in HL60 compared to HL60/MRI. Thus, the approximately 2-fold greater rate of differentiation of HL60/MRI appears related to the level of c-myc mRNA and not to the rate of turnover.
3. Transcripts of c-fms mRNA are detected during RA-induced granulocytic differentiation of HL60, but they do not increase in proportion to the extent of differentiation. However, c-fms mRNA in HL60/MRI is detectable 12 hours after exposure to RA and increases in amount as the extent of differentiation increases. Thus, it appears that c-fms is associated with monocytic differentiation, providing a positive support to reports that c-fms is related to the receptor for CSF-1, the macrophage colony stimulating factor.
4. During the monocytic differentiation of RA-induced HL60/MRI, c-fos is activated, not at an early stage of induction as is observed in HL60 induced with TPA, but at 1-2 days. However, even during granulocytic differentiation of HL60 induced with either RA or DMSO, the expression of c-fos is observed when HL60 cells are induced in a fetal bovine serum (FBS)-free defined medium supplemented with transferrin and insulin. No activation of c-fos is observed during differentiation of HL60 induced with RA or DMSO in FBS-supplemented medium. TPA induces the expression of c-fos at an early stage of induction,



whether or not FBS is present. Based on the expression of a mature myelomonocyte-specific surface antigen and superoxide production, the induction of HL60 with RA is more effective in serum-free medium than in serum-supplemented medium. In contrast, the differentiation of HL60 with DMSO is more effective in serum-containing medium. These results indicate that the expression of c-fos is not necessarily related to either monocytic or granulocytic differentiation of HL60, and that factor(s) in serum may modify c-fos expression during RA- or DMSO-induced granulocytic differentiation of HL60.

#### Proposed Course

1. Most studies on proto-oncogene expression rely on measurement of oncogene mRNA transcripts. The availability of specific antibodies to oncogene proteins should permit more detailed study of how the ultimate product, the oncogene proteins, change during differentiation. Two techniques will be used to measure specific oncogene proteins: Western blots and flow cytometry. By applying flow cytometry to this study it should be possible to correlate changes in oncogene proteins in individual cells with other differentiation-associated changes as well as other parameters such as cell cycle stage.
2. An understanding of why the HL60/MRI transplantable tumor does not respond in vivo to RA treatment will be investigated in greater detail. In the previous negative results the tumor was grown i.p. and RA was injected i.p. It is possible that RA is transported very rapidly from this compartment and does not return at concentrations high enough to induce differentiation of the cells. With the use of radioactive RA this possibility can be checked. In addition, inoculation of the tumor cells s.c. will be investigated as an alternative site for tumor growth. This site may have the advantage that the relative rate of tumor growth can be more easily measured.

#### Publications.

1. Breitman, T.R., Hemmi, H., Imaizumi, M.: Induction by physiological agents of differentiation of the human leukemia cell line HL60 to cells with functional characteristics. Prog. Clin. Biol. Res. 226: 215-233, 1986.
2. Hemmi, H. and Breitman, T.R. Combinations of recombinant human interferons. and retinoic acid synergistically induce differentiation of the human promyelocytic leukemia cell line HL60. Blood 69: 501-507, 1987.
3. Imaizumi, M., Uozumi, J., and Breitman, T.R. Retinoic acid-induced monocytic differentiation of HL60/MRI, a cell line derived from a transplantable HL60 tumor. Cancer Res. 47: 1434-1440, 1987.
4. Haces, A., Breitman, T.R. and Driscoll, J.S. Chemical differentiating agents. Differentiation of HL-60 cells by hexamethylenebis[acetamide] analogues. J. Med. Chem. 30: 405-409, 1987.



5. Imaizumi, M. and Breitman, T.R. Retinoic acid-induced differentiation of the human promyelocytic leukemia cell line, HL-60, and fresh human leukemia cells in primary culture: a model for differentiation inducing therapy of leukemia. Eur. J. Haematol. 38: 289-302, 1987.
6. Spruce, L.W., Rajadhyaksha, S.N., Berline, K.D., Gale, J.B., Miranda, E.T., Ford, W.T., Blossey, E.C., Verma, A.K., Hossain, M.B., van der Helm, D. and Breitman, T.R. Heteroarotinoids: synthesis, characterization, and biological activity in terms of an assessment of these systems to inhibit the induction of ornithine decarboxylase activity and to induce terminal differentiation of HL-60 cells. J. Med. Chem., 1987, in press.
7. Breitman, T.R. Retinoic acid-induced differentiation of HL60: Studies in vitro and in vivo. In Aarbakke, J., Chiang, P.K., Koeffler, H.P. (Eds.): Tumor Cell Differentiation: Biology and Pharmacology. Clifton, New Jersey, The Humana Press, Inc., 1987, in press.
8. Breitman, T.R. The role of prostaglandins and other arachidonic acid metabolites in the differentiation of HL-60. In Garaci, E., Paoletti, R., Santoro, M.G. (Eds.): Prostaglandins in Cancer Research. Berlin, Springer-Verlag, 1987, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 03580-18 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemical Research in the Development of New Anticancer Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

J. S. Driscoll Chief LMC, NCI

Others: V. E. Marquez Visiting Scientist LMC, NCI

## COOPERATING UNITS (if any)

Pharmacology Branch, DTP, NCI

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: PROFESSIONAL: OTHER:  
0.2 0.2

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects  (b) Human tissues  (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Ara-AC, a drug synthesized in this laboratory, is about to begin Phase I clinical trials in three institutions.

A patent application describing the antiviral activity of 4-deaminocytidine (zebularine) was filed.

A hexamethylene bis-hydantoin, which was 10 times more potent than HMBA in differentiating HL-60 cells, was the subject of a patent application.

Cyclopentenyl cytosine (CPE-C) has excellent preclinical antitumor activity as well as potent activity against both DNA and RNA viruses. A patent application was filed on this potential clinical antitumor candidate.



Project Description:General Objective:

The objective of this project is the discovery of new types of drugs which are clinically useful against cancer. Medicinal chemical research is directed toward the synthesis of new compounds which have potential as useful agents. Leads for this program are generated from structure-activity studies, the DTP screening program, the literature, and biochemical rationale.

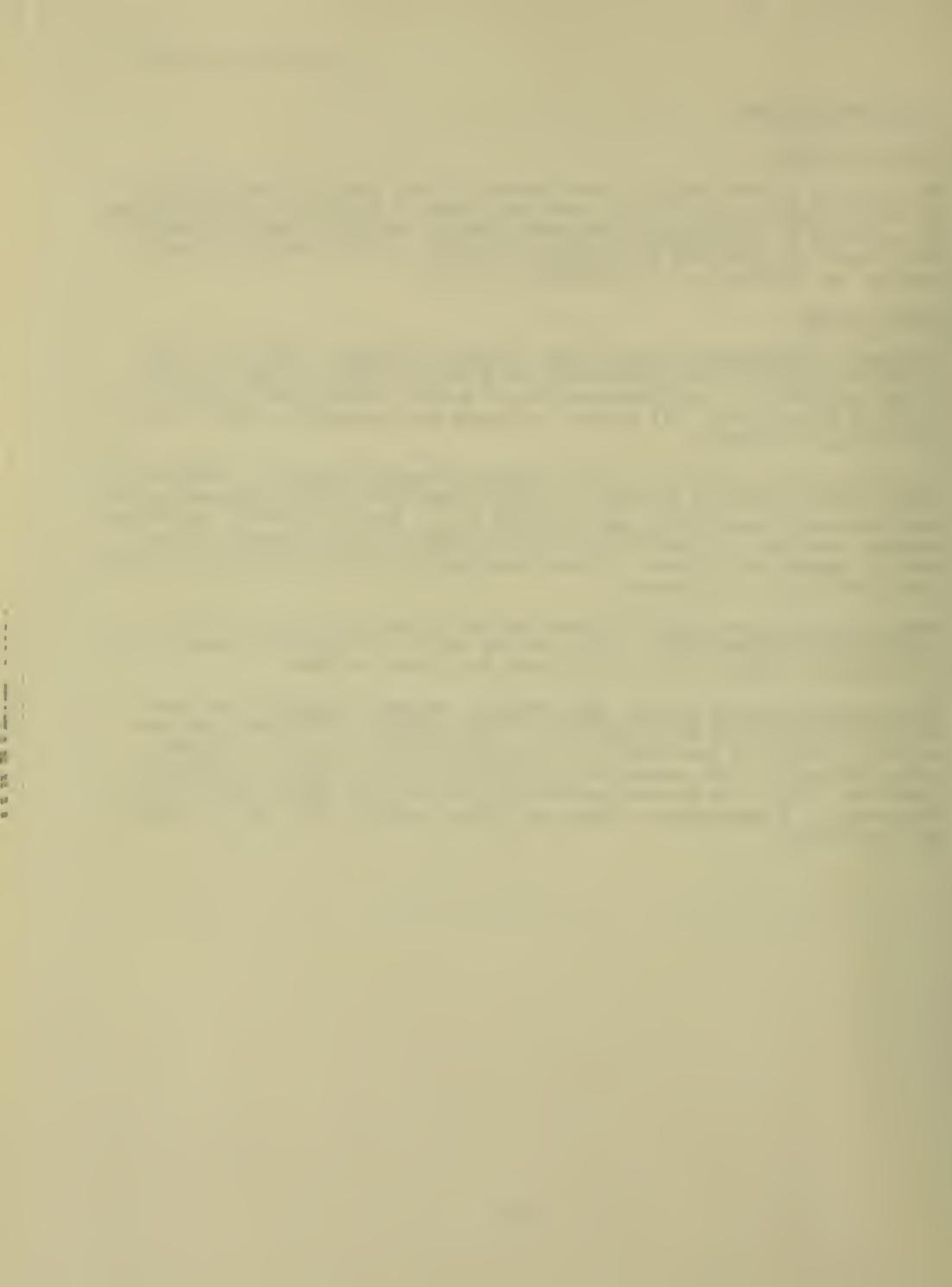
MAJOR FINDINGS

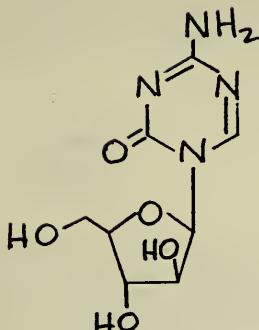
Arabinosyl-5-azacytosine [ara-AC] (Drs. Driscoll, Plowman): Ara-AC, a drug synthesized earlier in this laboratory, is scheduled to begin Phase I clinical trials during FY 87. Continued pre-clinical testing confirms the fact that ara-AC is active against all three of the human tumor xenografts used by the NCI for antitumor evaluation.

4-Deaminocytidine [zebularine] (Drs. Marquez, Plowman, Driscoll): Zebularine is the pyrimidine analog of the natural product, nebularine. It was synthesized in this laboratory several years ago and found to be a good inhibitor of the enzyme cytidine deaminase. In addition, it is active against L1210 leukemia and has activity against DNA viruses. A patent application was filed covering the patentable activity of this compound.

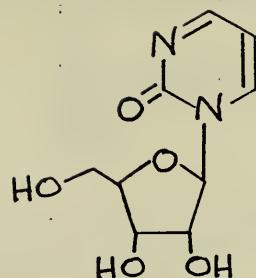
HMBA analogs (Dr. Driscoll): A patent application was filed on the new hexamethylenebisacetamide (HMBA) analogs synthesized as differentiating agents. The bis-hydantoin compound (1) is ca. 10 times more potent as HMBA.

Cyclopentenyl cytosine [CPE-C] (Drs. Marquez, Plowman, Driscoll): Antitumor testing continues with this potential clinical candidate. CPE-C has DN2 level activity in multiple tumor models. The antitumor activity of this compound is being studied in direct comparison with 3-deazauridine, another CTP synthesis inhibitor. CPE-C also has very potent antiviral activity against both DNA and RNA viruses. A comprehensive patent application covering CPE-C and its analogs has been filed.

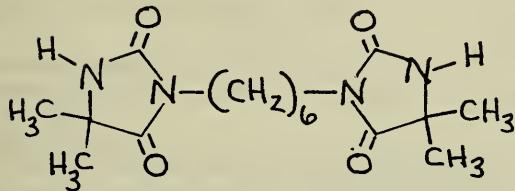




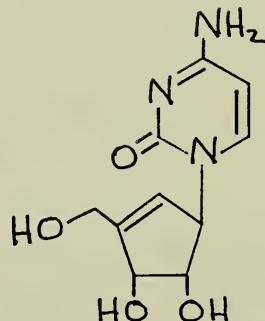
ara-AC



Zebularine



1



CPE-C

## PUBLICATIONS

1. Marquez, V.E., Driscoll, J.S., Johns, D.G.: Biological Activity of Pyrimidin-2-ones. U.S. Patent Application SN 940,273, filed December 8, 1986.
2. Driscoll, J.S., Haces, A., Breitman, T.R.: Chemical Differentiating Agents. NIH Patent Case #E-121-87; June 1987.
3. Marquez, V.E., Driscoll, J.S., Lim, M-I., Tseng, C.K-H., Haces, A., Glazer, R.I.: 3-Deazaneplanocin A and Method of Preparation. U.S. Patent SN 867,583, filed May 27, 1986.
4. Driscoll, J.S.: Cyclopentenyl Cytosine (CPE-C). A Neplanocin Analogue With Preclinical Antitumor and Antiviral Activity. Proceedings of the 1986 Beijing Symposium on Cancers in the Peoples Republic of China, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06173-02 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Dideoxynucleosides as Potential Anti-AIDS Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

John S. Driscoll Chief LMC, NCI

Others: V. E. Marquez	Visiting Scientist	LMC, NCI
C. Tseng	Senior Staff Fellow	LMC, NCI
S. Treanor	Chemist	LMC, NCI
R. Fuller	Chemist	LMC, NCI

## COOPERATING UNITS (if any)

Office of the Associate Director, Clinical Oncology Program, DTP, NCI

Laboratory of Biochemical Pharmacology, DTP, DCT, NCI

Drug Synthesis and Chemistry Branch, DTP, DCT, NCI

## LAB/BRANCH

Laboratory Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

3.4

1.9

1.5

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither

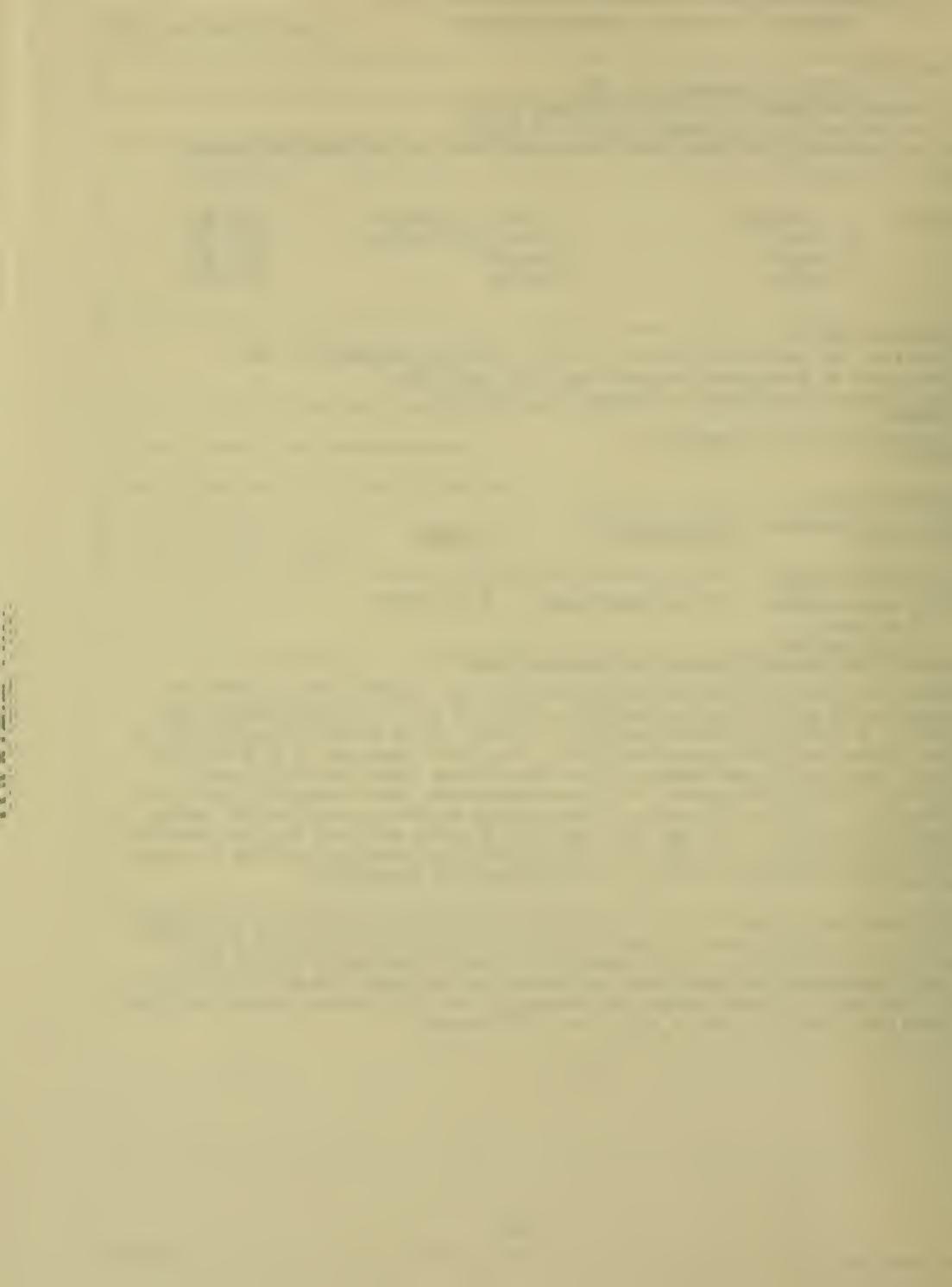
(a1) Minors

(a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dideoxypyrimidine and purine nucleosides have been synthesized as agents to protect ATH8 cells from the cytopathic effect of the virus which causes AIDS (HIV). 5-Fluoro-2',3'-dideoxycytidine (5-F-ddC) is as potent and protective as the parent drug, dideoxycytidine (ddC). The fluoro compound is 30 times less basic than the ddC and appears to have about three times the oral bioavailability of ddC in the mouse. All other comparisons show essentially identical properties. Toxicity studies will be designed to determine if 5-F-ddC has an advantage over ddC. 5-F-ddC also should be a useful PET scan drug for studying the pharmacology of ddC. Several other pyrimidine dideoxynucleosides have been prepared for anti-AIDS testing and pharmacological evaluation.

Dideoxyadenosine (ddA) is an anti-AIDS drug scheduled for clinical trial during FY 87. It is very unstable under acidic conditions, making an oral formulation of the drug difficult. 2',3'-dideoxy-2'- $\beta$ -fluoroadenosine (2'-F-ddA) has been synthesized and found to be just as active and potent as ddA in HIV tests. In addition, 2'-F-ddA undergoes no decomposition after 24 hours under the acidic conditions which give ddA a half-life of 35 seconds.



Project Description:General Objective:

The objective of this project is the discovery of 2',3'-dideoxynucleoside analogs superior to known inhibitors of the AIDS virus.

Specific Objectives:

1. Synthesis of pyrimidine analogs
2. Synthesis of acid-stable purine analogs

MAJOR FINDINGS

Synthesis of Dideoxypyrimidine Nucleosides (Drs. Marquez, Kim, Driscoll, Ms. Treanor, Mr. Fuller): 5-Fluoro-2',3'-dideoxycytidine (1a) has been synthesized and found to have properties similar to those of its parent, ddC (1b). Because of the fluorine atom, (1a) is less basic by 1.7 pK units than ddC and has significantly greater orally bioavailable in the mouse (63% vs 25%). All other properties of 5-F-ddC appear to be almost identical with ddC (CSF penetration, anti-HIV activity and potency).

5-Fluoro-2',3'-dideoxycytidinene (2) has been synthesized but has not yet been evaluated by Broder and Mitsuya in their test system.

5-Fluoro-2',3'-dideoxy-3'- $\alpha$ -methoxycytidine (3) was inadvertently prepared during the synthesis of compound 2. Surprisingly, this compound has anti-HIV activity and provides a new lead compound.

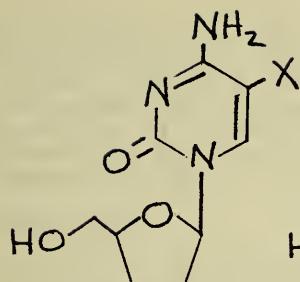
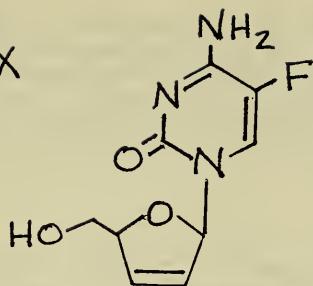
2',3'-Dideoxyuridine (4) was synthesized for biochemical and metabolic studies and provided to the Laboratory of Biochemical Pharmacology. As expected, (4) was not an active anti-HIV agent.

The synthesis of the 5'-phosphate dimer (5), suggested as a prodrug form for ddC by Dr. Broder, is in its final stage. Mass spectral conformation of the structure will initiate anti-HIV testing.

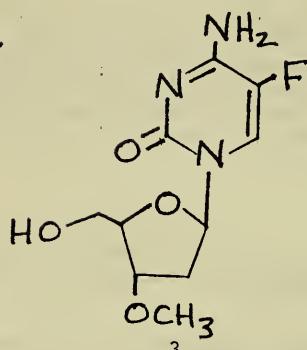
Synthesis of Acid-Stable Dideoxypurine Nucleosides (Drs. Marquez, Tseng, Driscoll): Dideoxyadenosine (ddA, 6) is an anti-AIDS clinical candidate. An IND is scheduled to be filed in late FY 87. Since this drug is very unstable under acidic conditions ( $t_{1/2} = 35$  seconds at pH 1, 37°C), there could be problems with an oral formulation which would expose ddA to stomach acid.

There are theoretical reasons to believe that a fluorine atom should stabilize ddA towards acid decomposition if it is placed in the 2'-position of the 2',3'-dideoxysugar. Two possible 2'-fluoro diastereomers are possible. Because an appropriate intermediate was readily available, we first synthesized 2'-F-ribo-ddA (7). While this compound is completely stable under acidic conditions, it is devoid of anti-HIV activity. Next, 2'-F-ara-ddA (8) was prepared by a

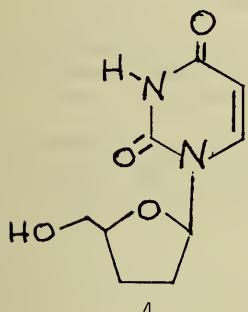


1a, X=F  
1b, X=H

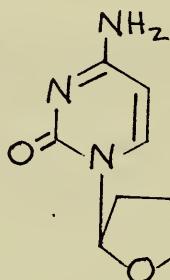
2



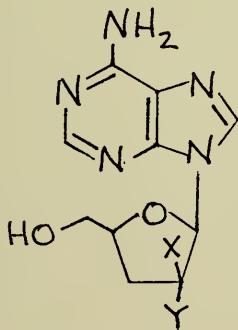
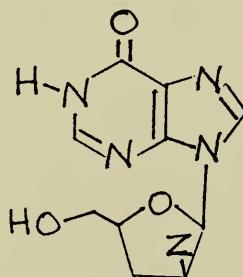
3



4



5

6, X=Y=H  
7, X=H; Y=F  
8, X=F; Y=H9, Z=F  
10, Z=H



14-step synthetic procedure. Compound 8 has the same potency and activity as its parent compound, ddA, and undergoes no change when subjected to pH 1 at 37°C for 24 hours. Compound 8 is deaminated by adenosine deaminase to the corresponding inosine analog (2'-F-ara-ddI, 9) which is anti-HIV active, and stable to acid. In addition, (9) is not a substrate for the catabolic enzyme, purine nucleoside phosphorylase (PNP) which destroys the anti-HIV activity of the non-fluorinated compound, ddi (10).

#### PUBLICATIONS

1. Kim, C.H., Marquez, V.E., Broder, S., Mitsuya, H., Driscoll, J.S.: Potential Anti-AIDS Drugs. 2',3'-Dideoxycytidine Analogues. J. Med. Chem. 30: 862-866, 1987.
2. Marquez, V.E., Tseng, C.K-H., Driscoll, J.S., Mitsuya, H., Broder, S., Roth, J.S., Kelley, J.A.: 2',3'-Dideoxy-2- $\beta$ -fluoroadenosine. An Acid-Stable Purine Nucleoside Active Against Human Immunodeficiency Virus (HIV). Biochem. Pharmacol., in press.
3. Driscoll, J.S., Marquez, V.E., Kim, C-H., Kelley, J.A.: 5-Substituted-2',3'-Dideoxycytidine Compounds with Anti-HTLV-III Activity. U. S. Patent Application SN 913,575, filed September 29, 1986.
4. Marquez, V.E., Driscoll, J.S., Tseng, C.K-H.: Acid-Stable Dideoxynucleosides Active Against the Cytopathic Effect of Human Immunodeficiency Virus. NIH Patent Case #E-258-87, filed April 17, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06174-02 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Cyclopentenyl Nucleoside Isosteres as Potential Antitumor and Antiviral Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Victor E. Marquez Visiting Scientist LMC, NCI

Others:	John S. Driscoll	Chief	LMC, NCI
	Christopher K-H. Tseng	Staff Fellow	LMC, NCI
	Sung K. Kim	Visiting Fellow	LMC, NCI
	Susan P. Treanor	Chemist	LMC, NCI
	Richard W. Fuller	Chemist	LMC, NCI

## COOPERATING UNITS (if any)

Laboratory of Biological Chemistry

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.6

## PROFESSIONAL:

1.3

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither

(a1) Minors

(a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This work continues to generate a series of new cyclopentenyl (CPE) nucleoside isosteres that are being studied as antitumor or antiviral agents. The discovery of the remarkable antitumor and antiviral activity of the cytosine analogue (CPE-C) has prompted an extensive investigation into this type of carbocyclic nucleoside. During this endeavor, new improved methods of synthesis and protection-deprotection procedures have been investigated and adapted to the chemistry of the CPE series.



Objectives:

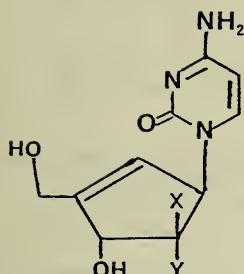
The objective of this work continues to be the systematic study of the structure-activity relationships (SAR) in this type of carbocyclic nucleoside through the alteration of the cyclopentenyl (CPE) moiety and the aglycon base in both the purine and pyrimidines series.

Major Findings:

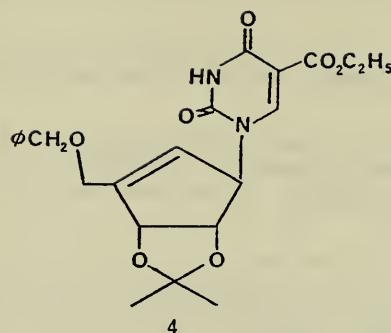
CPE-pyrimidine analogues. (Dr. Marquez, Dr. Kim, Ms. Treanor and Mr. Fuller): Syntheses of 2'-deoxycyclopentenyl cytosine (2'-d-CPE-C, 3) and aracyclopentenyl cytosine (ara-CPE-C, 2) have been completed. Both syntheses started with the suitably protected cyclopentenyl uracil (4) which after eight and nine steps, respectively, yielded the corresponding ara and 2'-deoxy analogues. This new synthesis of ara-CPE-C confirmed that our previous one-step synthesis of this compound gave a different product. The elucidation of the structure of the unknown isomeric compound is under investigation. Preliminary studies on the cytotoxicity of these compounds have been completed for the ara isomer and they indicate that the compound lacks activity against in vitro L1210 leukemia. Studies on the 2'-deoxy-analogue are underway. During the course of these investigations large amounts of cyclopentenyl uracil (CPE-U, 5) were prepared for biological studies. This compound appears to be an effective inhibitor of uridine-cytidine kinase in vivo in a manner that correlates with its in vitro activity. This appears to be an important finding since only a few of the inhibitors that are effective in vitro show activity when tested in vivo (Dr. J. Moyer, LBC, NCI, personal communication). Modification in the procedures for the scale-up synthesis of CPE-U resulted in significant improvements in the selection of deblocking methods to remove the benzyl protecting group at the 6'-position in this series of compounds. The method finally selected was that of Fuji et al. (Chem. Pharm. Bull. 28: 3662, 1980) which gave yields greater than 85% the fully deblocked carbocyclic moiety.

CPE-purine analogues (Dr. Tseng, Dr. Marquez and Mr. Fuller): 3-Deazaneplanocin (CPE-3-deazaadenine, 6) was resynthesized and submitted for an extensive evaluation against RNA viruses. Complete results are not available yet. Other purine derivatives have been prepared in collaboration with Dr. David Haines (1985-86 IPA visiting professor) from Wellesley College, as potential antitumor or antiviral agents. The target compounds include CPE-guanine (7), CPE-thioguanine (8), CPE-hypoxanthine (9), CPE-6-methoxy-2-aminopurine (10), and CPE-2,6-diaminopurine (11). All these new compounds have been characterized by high resolution NMR and FAB MS in our laboratory. Antiviral and antitumor testings are in progress.

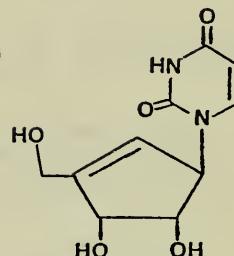




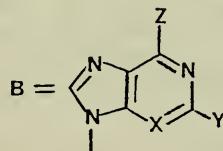
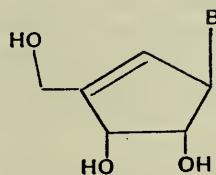
1, X=H; Y=OH (CPE-C)  
 2, X=OH; Y=H  
 3, X=Y=H



4



5



6, X=C; Y=H; Z=NH<sub>2</sub>  
 7, X=N; Y=NH<sub>2</sub>; Z=OH  
 8, X=N; Y=NH<sub>2</sub>; Z=SH  
 9, X=N; Y=H; Z=OH  
 10, X=N; Y=NH<sub>2</sub>; Z=OCH<sub>3</sub>  
 11, X=N; Y=Z=NH<sub>2</sub>



PUBLICATIONS

1. Glazer, R.I., Cohen, M.B., Hartman, K.D., Knodel, M.C., Lim, M-I., and Marquez, V.E.: Induction of differentiation in the human promyelocytic leukemia cell line HL-60 by the cyclopentenyl analogue of cytidine. Biochem. Pharmacol., 34: 2535-2539, 1985.
2. Moyer, J.D., Malinowski, N.M., Treanor, S.P., Marquez, V.E.: Antitumor and Biochemical Effects of Cyclopentenyl Cytosine. Cancer Res. 46: 3325-3329, 1986.
3. Glazer, R.I., Knodel, M.C., Tseng, C.K-H., Haines, D.R., Marquez, V.E.: 3-Deazaneplanocin A: A new inhibitor of S-adenosylhomocysteine synthesis and its effects in human colon carcinoma cells. Biochem. Pharmacol. 35: 4523-4527, 1986.
4. Glazer, R.I., Hartman, K.D., Knodel, M.C., Richard, M.M., Chiang, P.K., Tseng, C.K.H., Marquez, V.E.: 3-Deazaneplanocin A: A new and potent inhibitor of S-adenosylhomocysteine hydrolase and its effects on human promyelocytic leukemia cell line HL-60. Biochem. Biophys. Res. Comm., 135: 688-694, 1986.
5. Haines, D.R., Tseng, C.K-H., Marquez, V.E.: Synthesis and biological activity of unsaturated carboacyclic purine nucleoside analogs. J. Med. Chem., 30: 943-947, 1987.
6. Marquez, V.E., Lim, M-I., Khan, M.S., Kaskar, B.: (4R,5R)-(-)-3-Benzylxy-methyl-4,5-O-isopropylidene-2-cyclopentenone. An optically active  $\alpha$ ,  $\beta$ -unsaturated cyclopentenone for the synthesis of Neplanocin A and other cyclopentene carbocyclic nucleosides. In Townsend, L.B. and Tipson, R.S. (Eds.): Nucleic Acid Chemistry. Improved and New Synthetic Procedures Methods and Techniques. Part 3. (in press)
7. Marquez, V.E., Lim, M-I., Markovac, A., Priest, M.A.: (-)-Neplanocin A. In Townsend, L.B. and Tipson, R.S. (Eds.): Nucleic Acid Chemistry. Improved and New Synthetic Procedures, Methods, and Techniques. Part 3 (in press).
8. Marquez, V.E., Tseng, C.K-H., Treanor, S.P., Driscoll, J.S.: Synthesis of 2',3'-dideoxycyclopentenyl carbocyclic nucleosides as potential drugs for the treatment of AIDS. (Nucleos. & Nucleot.) (in press)



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06175-02 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis and Properties of Oligonucleotides Containing 5-azacytosine Residues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Victor E. Marquez

Visiting Scientist

LMC, NCI

Others: Amanda Goddard

Visiting Fellow

LMC, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.1 PROFESSIONAL: 1.1 OTHER:

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither

(a1) Minors

(a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This work has progressed to the stage that the synthesis of a 5-azacytosine containing dimer is possible. The reactivity and stability of the reduced phosphoramidite of 2'-deoxy-dihydro-5-azacytidine has been investigated and found to be compatible with the phosphoramidite chemistry used in automated DNA synthesizers. In a model experiment to test our approach, a dimeric structure containing dihydro-5-azacytosine and thymine was prepared and successfully oxidized to the desired dimer containing the required 5-azacytosine moiety. The ease with which this procedure works allows one to exchange cytosine residues for 5-azacytosine moieties in the construction of modified DNA molecules.

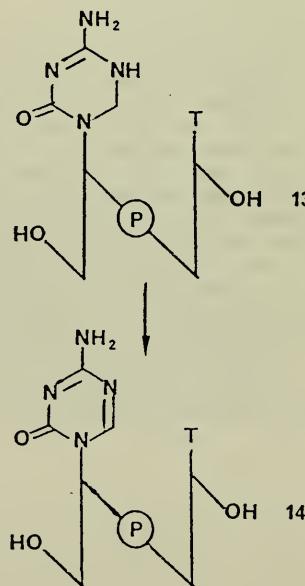
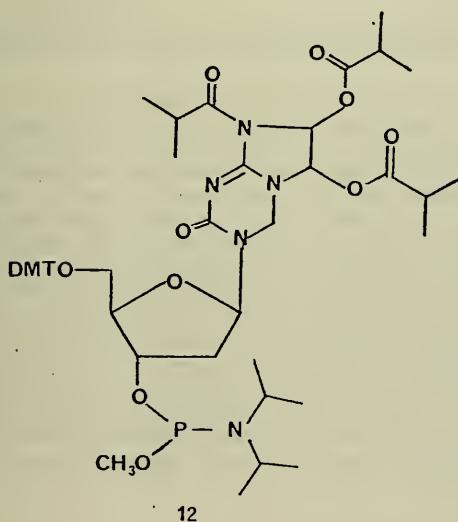


Objectives:

The long range objective of this project is the incorporation of 5-azacytosine residues into CG rich regions of promoter DNA sequences, with the idea of selectively inhibiting DNA methyltransferase and achieving activation of specific genes. A more immediate objective is the development of synthetic methods that will allow the execution of this project using current synthetic methodology for automated DNA synthesis.

Major Findings:

This project which began last year, has advanced significantly. After many attempts, a stable and reactive phosphoramidite synthon of the precursor 2'-deoxy-5,6-dihydro-5-azacytidine (12) has been synthesized. This compound reacted smoothly with other 3'-protected nucleosides to give phosphodiester dimers (e.g. DHAC-p-T, 13) in very high yield. After removal of all the protective groups, oxidation of the triazine ring to the desired 5-azacytosine moiety was efficiently performed with the aid of oxygen and a silicating reagent in refluxing acetonitrile to give 14. This novel procedure circumvents the intrinsic base instability of the 5-azacytosine moiety that precludes its direct use in the synthesis of DNA. Only after the DNA is assembled and isolated free of protective groups is the final oxidation performed. We are currently in the process of scaling-up our synthesis of phosphoramidite in order to use it in the automated DNA synthesizer.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CM 06176-02 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Enzyme Inhibitors as Potential Anticancer and Antiviral Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Victor E. Marquez Visiting Scientist LMC, NCI

Others: John S. Driscoll Chief LMC, NCI  
Christopher K.H. Tseng Staff Fellow LMC, NCI  
Chong-Ho Kim Visiting Fellow LMC, NCI  
Richard W. Fuller Chemist LMC, NCI

## COOPERATING UNITS (if any)

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Drug Synthesis and Chemistry Branch, NCI

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: PROFESSIONAL: OTHER:  
0.5 0.4 0.1

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects  (b) Human tissues  (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This work continues to explore the utility of mechanism-base inhibitors against enzymes critical for cancer chemotherapy. Successful inhibitors of inosine monophosphate dehydrogenase have been developed and future *in vivo* studies will be scheduled as soon as larger quantities of drugs become available. From the studies with our developed cytidine deaminase inhibitors, a better understanding of the enzyme's mechanism is being pursued through the analysis of the X-ray structure of the enzyme-drug complexes. In addition, drug combination studies with 5-fluoro-2'-deoxycytidine may open a new window of opportunity for these CDA inhibitors as adjuvants for cancer chemotherapy. These studies with CDA inhibitors are being performed in collaboration with academic institutions. The successful synthesis of the monomeric phosphonate adenosine nucleoside also paves the way for the synthesis of the corresponding oligoadenylyate trimer which, in addition to its increased lipophilicity, should be completely resistant to phosphodiesterase degradation. Our initial results in the synthesis of purine nucleoside phosphorylase inhibitors will be extended to provide an in-depth structure-activity study now that the major synthetic problems have been solved.



Objectives:

The objective of this project is to design mechanism-based inhibitors of various enzymes of interest in antitumor or antiviral chemotherapy. Our current approach is based on the construction of a modified substrate which will either inactivate the enzyme, bind tighter to its active site, or resist enzymatic degradation.

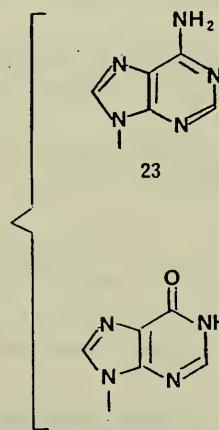
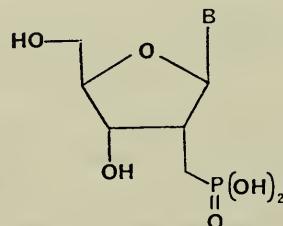
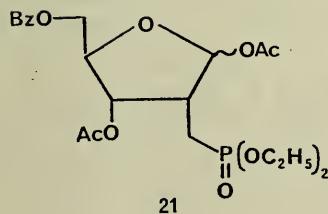
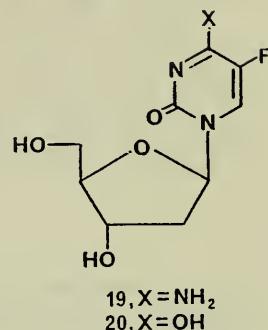
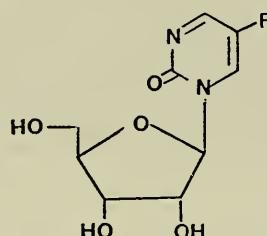
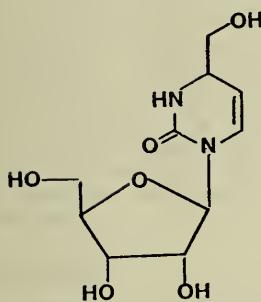
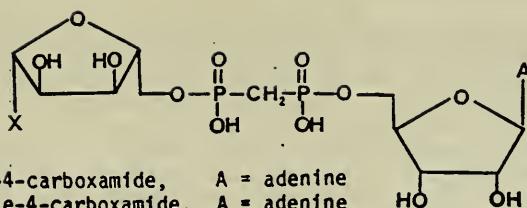
Major Findings:

Dinucleotide Analogues of NAD as IMP Dehydrogenase Inhibitors (Drs. Marquez, Tseng and Mr. Fuller): The detailed synthetic procedures of the hydrolytically stable dinucleotides betamethylene TAD (15) and beta-methylene SAD (16) have been submitted to a contractor (Starks Associates, Buffalo, NY) for a scale-up synthesis. Complete *in vivo* studies are being planned for these two important compounds as soon as they become available.

Cytidine Deaminase Inhibitors (Drs. Marquez, and Kim): Most of the synthetic work that relates to this project has been completed. We are collaborating with two important laboratories who are currently using our compounds 17 and 18 to a) investigate the crystalline structure of the enzyme bound to its substrate (Drs. Lloyd Frick and Richard Wolfenden, University of North Carolina) and b) to study the effects of these inhibitors in combination with 5-fluoro-2'-deoxycytidine (FdCR, 19) as a mechanism of selectively generating 5-fluoro-2'-deoxyuridine (20) as the monophosphate (FdUMP) in tumor cells (Dr. Sheldon Greer, University of Miami School of Medicine). The rationale for this combination is based on the premise that CDA inhibition will protect FdCR from systematic deamination allowing it to reach the intracellular compartment intact. Inside the cell, after its phosphorylation to FdCMP, it will be deaminated by dCMP deaminase which is significantly more abundant in tumor cells than normal cells. In the first project, a useful crystal structure of the enzyme bound to 5-fluoro-2-oxo-pyrimidine riboside has been resolved by X-ray analysis. Both studies are in progress.

Synthesis of Phosphonate Analogues of the 2',5'-Oligoadenylate Trimer as Stable Inducers of Interferon Production (Drs. Tseng and Marquez): The successful synthesis of the monomeric phosphonate 23 has been accomplished. The key step in this synthesis was the regioselective glycosylation of the phosphonate sugar 21 (prepared in 9 steps) from diacetone-D-gulose, (22) and 6-chloropurine. The stereochemistry of the product was ascertained by  $^1\text{H-NMR}$  NOE studies. Conversion of the 6-chloropurine analogue to the desired adenosine was easily accomplished by ammonolysis. Our efforts are now concentrated in the easier task of preparing the target trimer. Preliminary experiments will be performed to obtain dimeric compounds first.







Synthesis of Purine Phosphonates as Inhibitors of Purine Nucleoside Phosphorylase (Drs. Tseng and Marquez): The successful synthesis of the phosphonate-adenosine 23 from the previous project permitted its use as the starting material to obtain the target compounds for this project. Initial attempts to prepare the inosine derivative 24 from 23 by enzymatic deamination failed. Later, in more detailed studies in collaboration with Dr. D. Cooney (LBP), it was determined that the phosphonate-adenosine nucleoside 23 was not a substrate for adenosine deaminase. Chemical deamination with nitrous acid, however, yielded the desired inosine phosphonate nucleoside 24 in good yield. Preliminary experiments indicate that the compound is not inhibiting the phosphorolysis of inosine by PNP which suggests that perhaps the 3'-hydroxyl may steer the phosphonate group away from its ideal position through the formation of a cyclic phosphonate. This could have happened as a result of the acidic conditions used in the deamination reaction which would favor formation of the cyclic phosphonate structure. Further studies are planned with a sample that has been treated with NaOH to insure that the phosphonate is not in its cyclic form. If this fails, synthesis of the 3'-deoxy analogue will be considered. Once this problem is resolved, the guanosine analogue will be prepared using a similar chemical approach.

#### PUBLICATIONS

1. Marquez, V.E., Tseng, C.K-H., Gebeyehu, G., Cooney, D.A., Ahluwalia, G.S., Kelley, J.A., Dalal, M., Fuller, R.W., Wilson, Y.A., Johns, D.G.: Thiazole-4-carboxamide adenine dinucleotide (TAD). Analogues stable to phosphodiesterase hydrolysis. J. Med. Chem. 29: 1726-1731, 1986.
2. Ahluwalia, G.S., Cooney, D.A., Marquez, V.E., Jayaram, H.M., Johns, D.G.: Studies on the Mechanism of Action of Tiazofurin (2- $\beta$ -D-ribofuranosyl-thiazole-4-carboxamide) VI. Biochemical and Pharmacological Studies on the Degradation of Thiazole-4-carboxamide Adenine Dinucleotide (TAD). Biochem. Pharmacol. 35: 3783-3790, 1986.
3. Kim, C-H., Marquez, V.E., Mao, D.T., Haines, D.R.: Synthesis of pyrimidin-2-one nucleosides as acid-stable inhibitors of cytidine deaminase. J. Med. Chem. 29: 1374-1380, 1986.
4. Kelley, J.A., Driscoll, J.S., McCormack, J.J., Roth, J.S., Marquez, V.E.: Furanose-pyranose isomerization of reduced pyrimidine and cyclic urea ribosides. J. Med. Chem., 29: 2351-2358, 1986.
5. Haines, D.R., Fuller, R.W., Ahmad, S., Vistica, D.G., Marquez, V.E.: Selective cytotoxicity of a system L specific amino acid nitrogen mustard. J. Med. Chem. 30: 542-547, 1987.
6. Kim, C.H., Marquez, V.E.: Synthesis of ring-expanded cytidine: Homocytidine. J. Org. Chem. (in press)
7. Sutton, P.A., Cody, V., Marquez, V.E.: Structures of two seven-membered ring pyrimidine nucleoside derivatives. Nucleos. & Nucleot. (in press).



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECTPROJECT NUMBER  
Z01 CM 03581-18 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## The Analytical Chemistry of New Anticancer Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

James A. Kelley Research Chemist LMC, NCI

Others: John S. Driscoll Chief LMC, NCI  
Jeri S. Roth Chemist LMC, NCI  
Harry Ford, Jr. Biotechnology Fellow LMC, NCI  
Lajos Hegedus Visiting Fellow LMC, NCI

## COOPERATING UNITS (if any)

Laboratory of Biochemical Pharmacology, DTP, DCT; Clinical Pharmacology Branch, Pediatrics Branch, COP, DCT; Invest. Drug Branch, CTEP, DCT

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

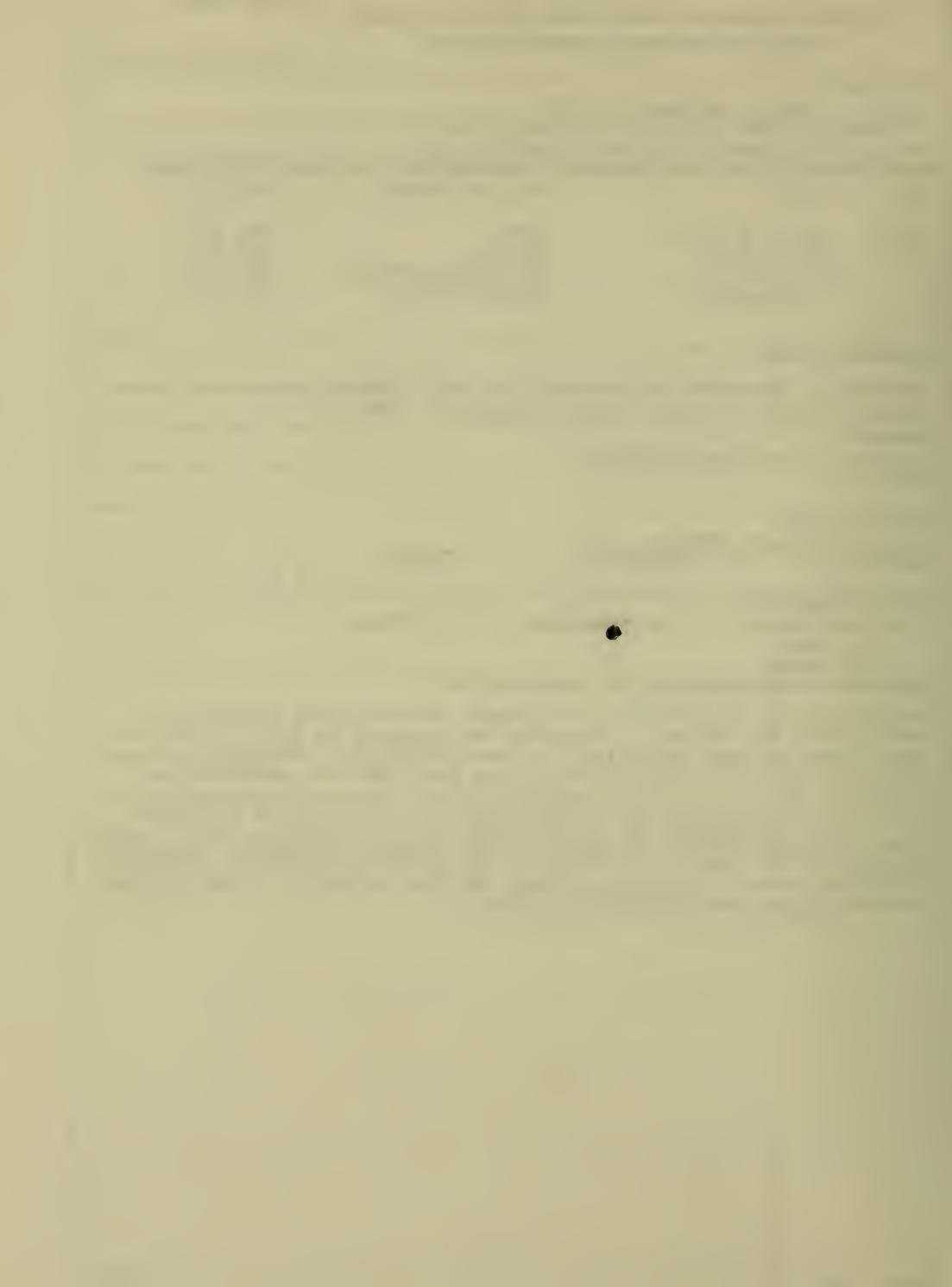
TOTAL MAN-YEARS: 2.0 PROFESSIONAL: 1.6 OTHER: 0.4

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects     (b) Human tissues     (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is the research and development of analytical methods which are used to: (1) establish the structure and purity of new anti-tumor agents and their metabolites, (2) determine physical and chemical properties of new anticancer drugs, (3) quantitate drugs and their metabolites in biological samples to elucidate pharmacology and to determine pharmacokinetics, and (4) study reaction mechanisms of potentially useful synthetic transformations. Mass spectrometry, gas chromatography and high-performance liquid chromatography, either alone or in combination, are emphasized techniques. Compounds of current interest are cytidine analogs, modified nucleosides, oligonucleotides, nitrogen mustards and differentiating agents.



Project Description:General Objectives:

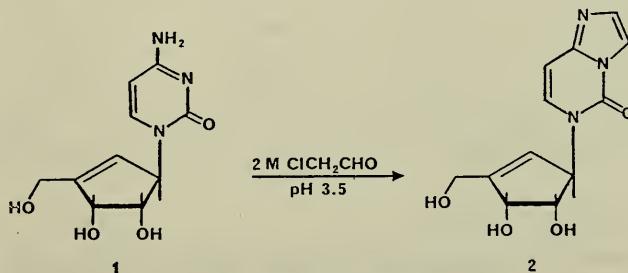
The objective of this project is the research and development of analytical techniques for establishing the structure and purity of new anticancer drug candidates, determining their important physical and chemical properties, elucidating structures of metabolites of new antitumor agents, measuring these drugs and their metabolites in physiological samples and studying reaction mechanisms. Mass spectrometry (MS), gas chromatography (GC), high-performance liquid chromatography (HPLC) and the combination of these techniques are the emphasized methods. Other analytical methods such as NMR, UV and IR spectroscopy are also employed.

Specific Objectives:

1. Analytical methods development and preclinical pharmacology for cyclopentenylcytosine (CPE-C).
2. Plasma and CNS pharmacokinetics of spiromustine in a pediatric Phase I clinical trial.
3. Plasma pharmacokinetics of arabinosyl-5-azacytosine (Ara-AC) in an adult Phase I clinical trial.
4. Oral bioavailability and metabolism of hexamethylene bisacetamide (HMBA) in human patients.

Major Findings:

1. Analytical Methods Development and Preclinical Pharmacology for Cyclopentenylcytosine (CPE-C) (Drs. Kelley, Hegedus): Cyclopentenylcytosine (CPE-C, NSC 375575, 1) is a synthetic cytosine nucleoside containing the same unsaturated sugar moiety as the antitumor antibiotic neplanocin A. Because of its antitumor and antiviral activity in several model systems and its potency as an inhibitor of CTP synthetase, this nucleoside is a potential candidate for development to clinical trial. A preliminary HPLC assay has been developed for the measurement of CPE-C in pharmaceutical vehicles and buffer solutions. This assay employs





reverse phase HPLC with UV detection at 280 nm and utilizes uridine as a hydrolytically stable internal standard. CPE-C (40  $\mu$ M) is completely stable at 37°C over a 24 hr period in pH 1, 6.5 and 7.4 buffers but slowly decomposes at pH 10, showing a 23% loss. CPE-C also binds tightly to a phenyl boronic acid solid phase extraction column, being eluted only at pH 1. This property will be utilized to isolate this compound from biological matrices. Fluorogenic derivatization of CPE-C through formation of compound 2 is being investigated to provide adequate sensitivity and specificity for HPLC detection. The above assay will be used in preclinical pharmacology and toxicology studies as well as in any clinical investigations.

**2. Plasma and CNS Pharmacokinetics of Spiromustine in a Pediatric Phase I Clinical Trial (Drs. Kelley, Heideman, Poplack, Ms. Roth):** Spiromustine (spirohydantoin mustard, NSC 172112) is a new lipophilic alkylating agent designed for the treatment of central nervous system (CNS) tumors. This compound is currently being evaluated in adult Phase II clinical trials. A cooperative pediatric Phase I study (NCI; Children's Hospital, Philadelphia; Children's Hospital National Medical Center, Washington, D.C.) in which 23 patients, 4 to 18 years of age, received spiromustine as an i.v. bolus dose on a weekly x 3 schedule every 28 days has been completed. The dose of spiromustine was escalated from 4.5 to 11.5 mg/m<sup>2</sup> per course of treatment until dose-limiting neurotoxicity was encountered. Plasma pharmacokinetics have been determined for 9 i.v. courses of therapy (6 patients) by measuring spiromustine in serial plasma samples using our previously developed capillary gas chromatography method employing thermionic detection. The plasma concentration versus time curve could be adequately

Table 1. Pharmacokinetic Summary of Spiromustine Pediatric Phase I Clinical Trial

Patient <sup>a</sup>	Dose (mg/m <sup>2</sup> )	Peak C <sub>p</sub> (ng/ml)	t <sub>1/2</sub> (α) (min)	t <sub>1/2</sub> (β) (min)	Clearance (ml/min/m <sup>2</sup> )
P.C.	5.5	215 <sup>b</sup>	1.5	9.3	2460
S.L.	6.5	556	.44	3.3	2660
A.S.	7.5	551	2.7	27.7	2300
J.C.	9.5	284	1.7	27.7	3050
T.P.	11.5	1920	1.3	19.6	1290
Mean Pediatric (n=9)	5.5-11.5		1.7 ± 0.71	15.9 ± 8.3	2134 ± 735
Mean Adult	3.1-5.5		.94 ± .26	12.5 ± 3.3	1650 ± 730

<sup>a</sup> only one patient at each dose level is represented

<sup>b</sup> measured; time of first blood sample varies



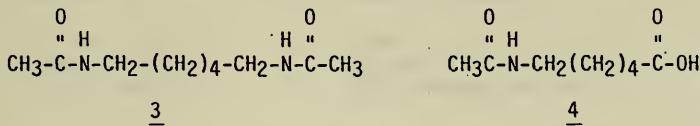
defined by a biexponential equation where the smaller rate constant corresponded to a  $t_{1/2}$  of  $15.9 \pm 8.3$  min. Although there was more variation in the calculated pharmacokinetic parameters for this pediatric trial (Table 1), they coincided closely with the results of a previous adult Phase I study. Observed levels of spiromustine in the cerebrospinal fluid (CSF) were surprisingly low. In a patient treated at the highest dose ( $11.5 \text{ mg/m}^2$ ), the drug could be detected in the CSF, but not measured. Subsequent administration of spiromustine as a short infusion (15 min) to maintain "high" plasma levels for a longer period of time, failed to enhance CSF levels. Thus the extensive plasma protein binding and rapid *in vivo* decomposition of spiromustine is probably reflected in its extremely low CSF to plasma ratio.

3. Plasma Pharmacokinetics of Arabinosyl-5-azacytosine (ARA-AC) in an Adult Phase I Clinical Trial (Drs. Kelley, Ford, Surbone, Cowan). Ara-AC (NSC 281272) is a new synthetic nucleoside which combines the structural elements of two established antitumor agents, Ara-C (the arabinose sugar) and 5-AC (the triazine base). Chemically Ara-AC behaves like 5AC, while it most closely resembles AraC in its antitumor and other biological properties. The plasma kinetics, disposition and metabolism of Ara-AC will be determined in human patients who will be given the drug as a 72-hr continuous i.v. infusion. Ara-AC will be measured in plasma, cerebrospinal fluid (if available), ascites fluid and urine by our previously developed and recently refined HPLC assay which has a limit of detection of less than  $50 \text{ ng/ml}$  ( $0.2 \mu\text{M}$ ) in human plasma.

4. Oral Bioavailability and Metabolism of Hexamethylene Bisacetamide in Human Patients (Drs. Kelley, Chun, Ward, Ms. Roth): Hexamethylene bisacetamide (NSC 95580, HMBA, 3), a potent *in vitro* differentiating agent has recently been evaluated in several Phase I clinical trials as a 5 or 10 day continuous i.v. infusion. Steady-state plasma concentrations ( $C_{ss}$ ) of 1-2 mM could be consistently produced in human patients by doses of HMBA greater than  $24 \text{ gm/m}^2/\text{day}$ . This matches drug concentrations required for tumor cell differentiation *in vitro*. However, a great deal of technical difficulty, inconvenience and expenditure of medical resources can be experienced with prolonged infusion schedules, so the efficacy of an alternate route of drug administration is being evaluated. A collaborative (NCI and Walter Reed Army Medical Center) Phase I clinical trial is being conducted to compare a 5 day period of oral administration of HMBA to a 120 hr continuous infusion in the same subjects. Since HMBA has a bitter taste which might inhibit direct oral intake, it is being given via nasogastric tube in equal doses every 4 hr. Oral bioavailability is being evaluated by comparing plasma pharmacokinetics, overall drug exposure (area under the concentration *versus* time curve or AUC), urinary elimination of parent compound and metabolite production. Both HMBA (3) and its major metabolite (6-acetamidohexanoic acid (4), will be measured in urine and plasma. Two patients have been evaluated at the initial dose of  $12 \text{ gm/m}^2/\text{day}$ , one-half the recommended Phase II dose for a 120-hr continuous infusion. In both cases a comparison of the oral and i.v. AUC indicates 100% bioavailability. The nadir of the HMBA plasma concentration following oral



dosing once a steady-state condition had been reached was about 50-60% of the  $C_{ss}$  during i.v. infusion, and the percentage of administered dose eliminated as parent compound ( $40 \pm 4\%$ ) was the same for i.v. or nasogastric administration



5. Synthetic and Collaborative Project Support (Dr. Kelley): Numerous samples which cannot be categorized as coming from any one project area were analyzed by the appropriate mass spectral and chromatographic techniques on an individual basis. Included in this group were carboacyclic nucleosides, diazepinone and ring-expanded nucleosides, dideoxyribose nucleosides, neplanocin analogs, choline analogs and myristylated peptides.

PUBLICATION:

1. Kelley, J.A., Driscoll, J.S., McCormack, J.J., Roth, J.S. and Marquez, V.E.: Furanose-pyranose isomerization of reduced pyrimidine and cyclic urea ribosides. J. Med. Chem., 29: 2351-2358, 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06177-02 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Analytical Chemistry of Anti-AIDS Agents

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

James A. Kelley Research Chemist LMC, NCI

Others: John S. Driscoll Chief LMC, NCI  
Jeri S. Roth Chemist LMC, NCI  
Harry Ford, Jr. Biotechnology Fellow LMC, NCI

## COOPERATING UNITS (if any)

Laboratory of Biochemical Pharmacology, DTP, DCT; Pediatric Branch, COP, DCT

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.0	0.6

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(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is the research and development of suitable analytical methods to: (1) establish the structure and purity of potential anti-AIDS agents and new antiviral drugs, (2) determine physical and chemical properties of these compounds and their metabolites, and (3) measure these drugs and their metabolites in biological samples to elucidate pharmacology and to determine pharmacokinetics. Gas chromatography, high-performance liquid chromatography and mass spectrometry are emphasized techniques. Compounds of current interest are dideoxycytidine, dideoxyadenosine, 2',3'-dideoxy-5-fluorocytidine, 2',3'dideoxy-2'- $\beta$ -fluoroadenosine, and 2',3'-dideoxy-2'- $\beta$ -fluoroinosine.



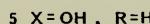
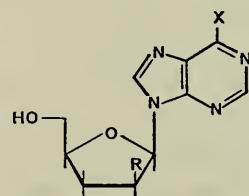
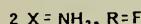
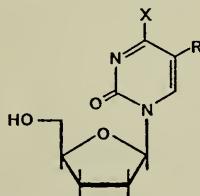
Project Description:General Objective:

The objective of this project is the research and development of suitable analytical methods for establishing the structure and purity of new anti-AIDS drug candidates, determining their important chemical and physical properties, elucidating structures of metabolites of these new agents, measuring these compounds and their metabolites in physiological samples and studying reaction mechanisms of synthetically important transformations. Gas chromatography (GC), high-performance liquid chromatography (HPLC) and mass spectrometry are the emphasized techniques. Other analytical methods such as NMR, UV AND IR spectroscopy, and ion exchange and affinity chromatography are also employed.

Specific Objectives:

1. Oral bioavailability of dideoxycytidine in mice.
2. Analytical methods development for 2',3'-dideoxyadenosine in pharmaceutical and biological matrices.
3. Comparative preclinical pharmacology of 2',3'-dideoxy-5-fluorocytidine in mice and monkeys.
4. Physical and chemical properties of sugar-modified dideoxynucleosides.

1. Oral Bioavailability of Dideoxycytidine in Mice (Drs. Kelley, Cooney, Johns, Ms. Roth): Preclinical pharmacology studies were continued on the high priority anti-AIDS drug, 2',3'-dideoxycytidine (ddC, 1). Since the *in vitro* effect of ddC is protective rather than curative, chronic *in vivo* administration might be indicated. Oral administration, if efficient and well-defined, could offer significant advantages over prolonged i.v. infusion. Accordingly, the oral bioavailability of ddC in mice was investigated in greater detail to see whether it was underestimated by the original area under the curve comparisons. The elimination of 100 mg/kg ddC oral and i.v. doses were followed using HPLC analysis and liquid scintillation counting. A 20% recovery of the i.v. bolus dose in,





the feces indicated substantial biliary excretion. Recovery of an even greater portion (56-60%) of the oral dose in the feces confirmed that ddC was incompletely absorbed. Comparison of the relative oral and urinary excretion indicated an oral bioavailability of only 28-33% and corroborated the earlier determination. Studies are in progress to define whether uptake mechanisms for oral absorption of ddC become saturated at high doses.

## 2. Analytical Methods Development for 2',3'-Dideoxyadenosine:

a. Rapid HPLC Assay for Dideoxyadenosine: (Dr. Kelley, Ms. Roth): Dideoxyadenosine (4, ddA) provides almost complete in vitro protection to ATH8 and H9 cells against the infectivity and cytopathic effect of the AIDS virus at drug concentrations of 5-50  $\mu$ M. Like ddC, ddA is a high priority clinical candidate. A rapid, sensitive and simple analytical method for ddA and its primary nucleoside metabolite, based on solid-phase extraction and reverse phase HPLC, has been developed. This assay is suitable for formulation and for in vivo toxicology and pharmacology studies. Sample preparation employs 2'-deoxycorformycin to inhibit deamination of ddA, N<sup>6</sup>-methyl-2'-deoxyadenosine to function as an internal standard to facilitate quantitation and a Sep-Pak C<sub>18</sub> cartridge to isolate ddA, internal standard and dideoxyinosine (5, dDI) metabolite from plasma or urine. An isocratic mobile phase (8.5% CH<sub>3</sub>CN/pH 6.8 0.01 M phosphate buffer) and UV detection at 260 nm is used to measure as little as 25 ng/mL (0.1  $\mu$ M) ddA with simultaneous detection of dDI metabolite in less than 20 min. The day-to-day assay precision shows a relative standard deviation of less than 3% for measuring 1  $\mu$ g/ml ddA over a two week period. Plasma samples can be stored frozen for at least two weeks without decomposition if adenosine deaminase is inhibited immediately with 2'-deoxycorformycin.

b. Solution and Plasma Stability of Dideoxyadenosine (Dr. Kelley, Ms. Roth): Dideoxyadenosine is very unstable in acidic solutions that approximate the pH of human gastric fluid (pH 1.2-1.7) and appears to be unsuitable for oral administration. The stability of both ddA and 2'-deoxyadenosine (2'-ddA) under acidic conditions is compared in Table 1. ddA also has a relatively short existence in RPMI 1640 culture media which is used to incubate HIV-infected ATH8 cells and

Table 1. Stability of ddA and 2'-dA Under Acidic Conditions (37°C)

pH	$t_{1/2}$ (min)			
	ddA		2'-dA	
	2 $\mu$ g/ml	10 $\mu$ g/ml	2 $\mu$ g/ml	10 $\mu$ g/ml
1	0.43	0.43	14.3	13.8
2	5.3	6.4	188	184



thus provide an in vitro test system for anti-AIDS activity. Ten micromolar ddA has a  $t_{1/2} = 1.1 \pm 0.1$  hr in the above system and is completely converted to ddI. Although this deamination can be completely blocked by 1  $\mu$ M 2'-deoxycoformycin, it is clear that ddA was not present after the first day in the original tests of anti-AIDS activity. Decomposition of ddA is even more rapid in rat plasma, having a  $t_{1/2}$  of only 8.7 min; an equally fast production of ddI is also observed. Human plasma shows considerable variability in decay rate from subject to subject, but like the rat, the decomposition of ddA can be blocked by 2'-deoxycoformycin.

c. Bolus Dose Plasma Kinetics (Drs. Kelley, Litterst, Ms. Roth): The HPLC assay of Section 2a was employed to determine the plasma kinetics of 50 mg/kg ddA given as an i.v. bolus to male Sprague-Dawley rats. The plasma elimination of ddA was exceedingly rapid (mean residence time = 53 sec; clearance = 834 mL/min/kg) and ddA could not be measured after 15 min. High levels of ddI were detected almost immediately, however, indicating that the in vivo conversion of ddA to ddI was extremely rapid. The subsequent plasma elimination of ddI exhibited typical biphasic kinetics with  $t_{1/2} (\alpha) = 5.9$  min and  $t_{1/2} (\beta) = 20$  min.

### 3. Comparative Preclinical Pharmacology of 2',3'-Dideoxy-5-fluorocytidine:

2',3'-Dideoxy-5-fluorocytidine (2, 5-F-ddC) is a synthetic analog of ddC, which possesses equivalent in vitro anti-AIDS activity. Chemically, 5-F-ddC is slightly more lipophilic and its 4-amino moiety possesses a lower pKa (2.5 versus 4.3) than dideoxycytidine. Two areas of concern about ddC based on previous preclinical pharmacology studies (see Section 1) have been its limited penetration of the central nervous system (CNS) and its incomplete oral bioavailability in mice. A targeted pharmacological evaluation of 5-F-ddC (because of very limited amounts of compound) with regard to these properties has therefore been initiated to see whether it offers any advantages over ddC itself.

a. Central Nervous System Pharmacokinetics of 5-F-ddC in Non-Human Primates (Drs. Kelley, Ford, Marquez, Poplack, Balis): The cerebrospinal fluid (CSF) and plasma kinetics of a 27 mg/kg (200 mg/m<sup>2</sup>) i.v. bolus dose of 5-F-ddC, a dose equivalent to previously studied doses of ddC, have been investigated in male rhesus monkeys with implanted Ommaya reservoirs. Slightly higher plasma levels are achieved with 5-F-ddC and CSF levels in excess of 2  $\mu$ M are observed. However, because of this greater plasma exposure, the CSF:plasma AUC ratio of 5-F-ddC ( $0.022 \pm .001$ ) is slightly less than that of ddC ( $0.033 \pm 0.007$ ). Measureable levels of the deaminated metabolite 5-F-ddU (3) are present in plasma and urine, but not CSF. This in vivo deamination appears to be equivalent to that observed for ddC with a plasma 5-F-ddU:5-F-ddC AUC ratio of  $0.12 \pm 0.03$ . Urinary excretion is also the major route of elimination for 2, with the majority of the dose being excreted in the urine as either parent compound or as metabolite 3 (about 10%). Therefore, 5-F-ddC is quite similar to ddC in all respects in this rhesus monkey model.

b. Oral Bioavailability Studies (Drs. Kelley, Ford, Litterst): The plasma kinetics of both an i.v. bolus and an oral dose of 33 mg/kg 5-F-ddC were determined and compared in male BDF<sub>1</sub> mice. Typical biphasic kinetics were seen for the i.v. bolus dose with a terminal  $t_{1/2}$  of 31 min. 5-F-ddC was rapidly absor-



bed from the gut after oral intubation with peak plasma levels of 10-11  $\mu\text{g/mL}$  being achieved in 15-30 min. A concentration plateau similar to that observed for oral ddC was also seen for oral 5-F-ddC after 3 hr, but it was not as pronounced. Comparison of the oral and i.v. AUCs indicated an apparent bioavailability of 63%, while comparison of urinary excretion data suggested a bioavailability as great as 87-89%. Although this exceeds the bioavailability of ddC in this species, a lower dose of 5-F-ddC (33 mg/kg vs 100 mg/kg) was used. Studies are also in progress to determine whether uptake mechanisms for oral absorption of 2 become saturated at higher doses (see Section 1).

#### 4. Physical and Chemical Properties of Sugar-Modified Dideoxynucleosides

(Drs. Kelley, Ford, Marquez, Tseng, Ms. Roth): The acid stability of a series of purine nucleosides incorporating a fluorine atom in the 2'- $\beta$ -position of the dideoxyribose sugar were evaluated and compared to ddA. 2',3'-Dideoxy-2'- $\beta$ -fluoroadenosine (6, 2'- $\beta$ -F-ddA), a ddA analogue with equivalent in vitro antiAIDS activity, was found to be completely stable in the pH range of the human stomach (pH 1-2) (Figure 1). Microscale enzymatic conversion of 6 to inosine 7 resulted in a dideoxynucleoside which was likewise stable to acid-catalyzed hydrolysis. A comparison of the deamination kinetics of 6 versus ddA using bovine adenosine deaminase indicated that ddA was a better substrate for the enzyme. Microscale purification using solid-phase extraction was employed to produce sufficient 7 for physical characterization and in vitro analysis of anti-AIDS activity.

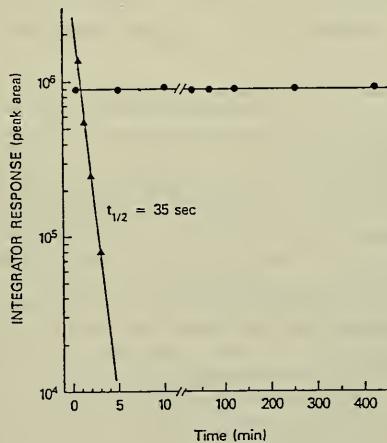


Figure 1. Concentration versus time profile for ddA (•) and 2'- $\beta$ -F-DDA ( $\Delta$ ) at pH 1 and 37°C. The time scale for the first 10 min is greatly expanded. Kinetics for both compounds were determined simultaneously.

#### Publication:

1. Kelley, J.A., Litterst, C.L., Roth, J.S., Vistica, D.T., Poplack, D.G., Cooney, D.A., Nadkarni, M., Balis, F.M., Broder, S. and Johns, D.G.: The disposition and metabolism of 2',3'-dideoxycytidine, an in vitro inhibitor of HTLV-III infectivity, in mice and monkeys. Drug Metab. Disp. (in press).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06178-02 LMC

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Applications of New Mass Spectral Techniques

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

James A. Kelley Research Chemist LMC, NCI

Others: Dong-Cheul Moon Visiting Fellow LMC, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Medicinal Chemistry

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: PROFESSIONAL: OTHER:  
1.2 1.2

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects  (b) Human tissues  (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is research on and development of new mass spectral techniques in order to provide new and/or more rapid solutions to problems involving (1) chemical structure determination, (2) complex mixture analysis and (3) measurement of trace components in biological systems. The utility and scope of these mass spectral methods are determined, and a comparison to other types of analysis, both new and established, is carried out. Fast atom bombardment mass spectrometry and combined liquid chromatography - mass spectrometry are the techniques of current interest. Fast atom bombardment mass spectrometry in both the positive and negative ion mode has been applied extensively to the rapid structure determination of base and sugar-modified nucleosides and synthetic nucleotides. A microscale desalting procedure to remove ionic contaminants which suppress sample ionization in fast atom bombardment mass spectrometry has been developed and applied to selected synthetic and biologically derived nucleosides. Studies of spectral anomalies generated by sample-matrix interactions during fast atom bombardment ionization continue.



Project Description:General Objective:

The objective of this project is the development and application of new mass spectral techniques for the rapid analysis of complex mixtures, measurement of trace components in biological systems and chemical structure determination. Fast atom bombardment mass spectrometry and combined liquid chromatography-mass spectrometry are the new techniques of current interest. The advantages and limitations of these new methods to already established techniques is also an area of concern.

Specific Objectives:

1. Rapid structural analysis of nucleosides and nucleotides by fast atom bombardment mass spectrometry.
2. Characterization of matrix-sample interactions to enhance fast atom bombardment mass spectrometry.

Major Findings:

1. Rapid Structural Analysis of Nucleosides and Nucleotides by Fast Atom Bombardment (FAB) Mass Spectrometry (Dr. Kelley): The LMC continues to have an extensive program in the synthesis of new nucleosides and nucleotides of novel structure. Rapid and simple methods employing FAB mass spectrometry have been developed and utilized to the characterize these nucleic acid constituents without derivatization. Positive ion FAB mass spectrometry provides rapid information on the identity of both the base and sugar in these new nucleosides. Stereochemical information is limited, however, since the positive ion primary FAB mass spectrum cannot differentiate between anomers or between structures isomeric in either the base or the sugar. Accurate mass measurement using glycerol matrix ions as internal mass references results in mass accuracies of  $\pm 0.002$  daltons (8 ppm) and allows determination of elemental compositions in those cases where insufficient sample exists for a classical elemental analysis. Negative ion FAB mass spectrometry has been applied to determine the structure and purity of chemically and enzymatically synthesized nucleotides using previously developed structural correlations. The enzymatically synthesized triphosphate of cyclopentenyl cytosine has been characterized and shown to contain a small amount of ADP, and the chemical synthesis of the dideoxycytidine-5'-monophosphate dimer has been monitored.

2. Characterization of Matrix-Sample Interactions to Enhance Fast Atom Bombardment Mass Spectrometry (Drs. Kelley, Moon): Fast atom bombardment mass spectrometry is a newer soft ionization technique that has found wide application in the analysis of thermally-sensitive, polar, high molecular weight materials. Because mass spectra from FAB ionization arise from ion-molecule reactions within the glycerol (or other) matrix, certain complications may be anticipated. The most common of these is that the spectrum of glycerol or matrix, including contaminants in the matrix, is superimposed on the spectrum of the sample. This is no problem if the matrix is well-characterized and chemically stable, since the spectrum of the matrix can serve as an internal mass marker and may be computer



subtracted. Of more concern is adduct formation due either to the matrix or to ionic contaminants in the sample. These may make molecular weight determination next to impossible by radically changing the spectrum or by completely suppressing ionization of the analyte as in the case of sodium ion contamination. Because sodium is ubiquitous in biological systems, a simple and rapid, yet efficient, microscale procedure for removing this and other cations from samples isolated from such sources is under development.

The effects of salt contamination on the FAB spectra of nucleosides have been determined and quantified. A log-linear relationship is observed between the abundance ratios of cationized to protonated ions and the molar ratio of salt to sample. Moreover, when there is ten-fold molar excess of salt (70% NaCl by weight), the positive ion FAB spectrum of the nucleoside is completely suppressed. This is a situation that frequently exists for samples of biologic origin that have been isolated by HPLC or column chromatography using an ionic buffer gradient, since even volatile buffers contain small amounts of non-volatile salts that become concentrated by lyophilization. Other significant sources of sodium ion contamination have been identified as ion leaching from glassware and dissolution of silica-based chromatography media when aqueous mobile phases are used. Most ( $98 \pm 1\%$ ) of this ionic contamination can be removed when it is at an equimolar level to sample by using a low pressure, high capacity, reverse phase minicolumn system. This system has been extensively evaluated using cytidine, the most difficult nucleoside to retain on a reverse phase column. At the  $2 \mu\text{M}$  level (480  $\mu\text{g}$  cytidine) with equimolar NaCl contamination, an absolute recovery of  $76 \pm 11\%$  is observed and the FAB spectra show insignificant cationization of the analyte ( $\text{M}^+/\text{M}+\text{Na}^+ > 20$ ). Very high salt concentrations require two passes through the minicolumn system. The usefulness of this procedure for microscale synthesis has also been demonstrated since it was employed to purify microgram amounts of enzymatically generated 2',3'-dideoxy-2'- $\beta$ -fluoroinosine. The application of this procedure to selected research problems is currently under investigation.

#### PUBLICATIONS:

1. Kelley, J.A., Tseng, C.K.H. and Marquez, V.E.: Applications of FAB/MS in the synthesis of NAD analogues and the structure determination of oligoribonucleotides. Adv. Mass Spectrom. 1985, Part B; 1571-1572, 1986.
2. Marquez, V.E., Tseng, C.K.H., Gebeyehu, G., Cooney, D.A., Ahluwalia, G.S., Kelley, J.A., Dalal, M., Wilson, Y.A. and Johns, D.G.: Thiazole-4-carboxamide adenine dinucleotide (TAD). Analogues stable to phosphodiesterase hydrolysis. J. Med. Chem. 29: 1726-1731, 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM-07102-12 LBP

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tubulin as a Site for Pharmacologic Attack

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	E. Hamel	Senior Investigator	LBP, NCI
Others:	R.-L. Bai C. Duammu G. J. Kang C. M. Lin	Visiting Fellow Visiting Fellow Visiting Fellow Biologist	LBP, NCI LBP, NCI LBP, NCI LBP, NCI

## COOPERATING UNITS (if any)

1) G.R. Pettit, Arizona State University; 2) L. Jurd, Dept. of Agriculture;  
 3) L. J. Powers, Ricerca Corp., Painesville, OH; 4) A. Brossi, NIDDK

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

1.9

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of this project is the development of new antineoplastic agents directed against tubulin, a protein critical for cell division. Work was continued with combretastatin congeners, a series of newly isolated natural products, more active than combretastatin itself. Several members of the series are among the most potent microtubule inhibitors yet described. The mechanism of action of 2,4-dichlorobenzyl thiocyanate was examined. The drug specifically alkylates the  $\beta$ -tubulin polypeptide, probably at a critical cysteine residue. Derivatives of 6-benzyl-1,3-benzodioxole continued to interest the laboratory because their facile synthesis permits a structure-function approach to the colchicine/podophyllotoxin binding site of tubulin. Derivatives of 5,6-diphenylpyridazin-3-one, which bind to a distinct site on tubulin (i.e., no competition with other drugs), continued to be evaluated in a search for maximally active agents. Alkyl carbamates of aromatic amines continued to be examined in a search for new anti-tubulin agents. Colchicine analogs with unusual structural features and/or biological properties were evaluated to quantitate their interactions with tubulin.



Microtubules, cellular organelles critical for cell division, are sensitive to a number of antineoplastic drugs. These drugs all cause cells to accumulate in metaphase, disrupting mitosis, for microtubules form the mitotic spindle. The major constituent of microtubules is an acidic protein known as tubulin, and it is the cellular target of virtually all antimitotic drugs. New antimitotic agents continue to be an active area of interest in the laboratory. We are currently studying the following classes of drugs:

- 1) Analogs of combretastatin. Combretastatin (NSC 348103) is a natural product isolated by G.R. Pettit of Arizona State University from the South African tree Combretum caffrum; and we have demonstrated that combretastatin is a potent inhibitor of tubulin polymerization and binds at the colchicine site of tubulin. Dr. Pettit's group has now purified and characterized about 20 additional compounds from Combretum caffrum and chemically synthesized an additional 20 compounds of related structure. Several analogs of combretastatin were also synthesized in our laboratory. At least six of these agents are considerably more potent than combretastatin itself as tubulin inhibitors. A detailed structure-activity study characterizing these drugs was performed.
- 2) The compound 2,4-dichlorobenzyl thiocyanate (NSC 145813) was previously shown to inhibit mitosis in murine leukemia cells and tubulin polymerization *in vitro*, and, in collaboration with other investigators, we demonstrated that certain lines of Chinese hamster ovary cells with a mutant  $\beta$ -tubulin gene were resistant to NSC 145813.
- 3) Radiolabeled NSC 145813 was prepared and found to form covalent bonds with tubulin, particularly with  $\beta$ -tubulin. At high drug concentrations (i.e., tubulin:drug = 1:10), multiple drug molecules form covalent bonds with a single tubulin molecule. Several lines of evidence demonstrate that NSC 145813 reacts with cysteine residues, forming disulfide bonds with the protein. No other class of antimitotic drug seems to interfere specifically with the interaction of NSC 145813 with tubulin.
- 3) A large number of derivatives of 6-benzyl-1,3-benzodioxole have been prepared by Dr. L. Jurd of the Department of Agriculture as potential insect sterilants. A significant number of these compounds have antineoplastic activity and inhibit tubulin polymerization. They are most analogous to podophyllotoxin structurally and, like podophyllotoxin, inhibit both tubulin-dependent GTP hydrolysis and the binding of colchicine to tubulin. Initial studies established minimal structural requirements for the simplest benzylbenzodioxole derivatives (a 1-3 carbon substituent at position 5, and a methoxy group at position 4' in the benzene ring) which have either an unsubstituted one carbon bridge between the benzene and benzodioxole rings or one or two methyl groups at this bridge position. Additional methoxy substituents on the benzene ring at the 3' and 5' positions, which seemingly increase the structural analogy to podophyllotoxin, resulted in almost complete loss of activity.

Dr. Jurd has also prepared a group of compounds with a third ring (of variable structure) fused to the benzodioxole moiety. The benzene ring is attached directly to this third ring. Several of these agents have strong antitubulin activity, and all of the compounds active against tubulin in



vitro also cause mitotic arrest. All compounds have three methoxy groups, attached at positions 3', 4' and 5', of the benzene ring. Analogs with a different methoxy substituent pattern have reduced activity. Although both the third fused ring and the trimethoxy structure appear to substantially increase their analogy to podophyllotoxin, these new agents are more comparable to colchicine in their effects on tubulin-dependent GTP hydrolysis; for, like colchicine, they stimulate rather than inhibit this reaction even while inhibiting the microtubule assembly reaction normally coupled to GTP hydrolysis.

- 4) Dr. L. J. Powers of Ricerca Corporation has prepared numerous derivatives of 5,6-diphenylpyridazin-3-one as potential antihypertensive agents. Some of these compounds were found to be potent herbicides as a consequence of inhibition of mitosis in plant tissues. Several members of this class were then submitted to the NCI for screening, and some of these were found to have antineoplastic activity. We have found that a number of these drugs inhibit mitosis in mammalian cells in culture and the polymerization of tubulin in vitro. They potently stimulate tubulin-dependent GTP hydrolysis; but they probably bind at a previously undescribed site on tubulin, for they do not inhibit the binding of either colchicine, vinblastine, maytansine or GTP to the protein. Active compounds possess a nitrile group at position 4; and in vitro interactions with tubulin are significantly enhanced by chloride substituents on the phenyl rings, both of which are required for antitubulin activity. There is little overlap between compounds most active against mammalian tubulin and those which are most active in inhibiting mitosis in plant cells. In collaboration with Dr. Powers we are continuing to study structure-activity correlations in this class of drugs to develop maximally active agents. One of the most active compounds has been prepared in a radiolabeled form, with the label in the phenyl rings. We were unable to demonstrate binding of the drug to tubulin. To resolve this paradox, the most active agent is being prepared with radiolabel in the pyridazinone ring.
- 5) A number of compounds with very different structures have been found to have antineoplastic and antimitotic properties and to inhibit tubulin polymerization. Their only common feature is that they are alkyl carbamates of aromatic amines. A computer search of the NCI drug collection produced over 140 compounds with promising structural features. These were screened for effects on tubulin-dependent GTP hydrolysis, and over fifty compounds were positive. These in turn were examined for effects on tubulin polymerization, and about a dozen drugs had significant inhibitory activity. These agents are being studied in further detail.
- 6) Dr. A. Brossi of the NIDDK has isolated and synthesized a large number of analogs of the classic microtubule inhibitor colchicine. Some of these with reduced antitubulin activity in vitro have recently been found to retain the substantial antiinflammatory activity of colchicine, suggesting that this property may not result from colchicine's antimitotic properties. Since the earlier characterization of the interactions of these agents with tubulin had not been definitive, we have undertaken a collaboration with Dr. Brossi to determine whether the antiinflammatory properties of colchicine indeed derive from a drug target other than tubulin. In addition, many of these colchicine analogs still need to have their interactions with tubulin



quantitated more precisely to provide possible structure-activity insights into a number of the unique characteristics of the colchicine-tubulin interaction (e.g., temperature-dependent, relatively slow, and irreversible binding of the drug to the protein).

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1. Batra, J.K., Lin, C.M., Hamel, E., Jurd, L. and Powers, L.J.: New antineoplastic agents with antitubulin activity. Ann. N.Y. Acad. Sci. 466: 785-787, 1986.
2. Abraham, I., Dion, R.L., Duanmu, C., Gottesman, M.M. and Hamel, E.: 2,4-Dichlorobenzyl thiocyanate, an antimitotic agent that alters microtubule morphology. Proc. Natl. Acad. Sci. USA 83: 6839-6843, 1986.
3. Batra, J.K., Jurd, L. and Hamel, E.: Morpholino derivatives of benzylbendioxole, a study of structural requirements for drug interactions at the colchicine/podophyllotoxin binding site of tubulin. Biochem. Pharmacol. 35: 4013-4018, 1986.
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5. Pettit, G.R., Singh, S.B., Niven, M.L., Hamel, E. and Schmidt, J.M.: Isolation, structure, and synthesis of combretastatins A-1 and B-1, potent new inhibitors of microtubule assembly, from Combretum caffrum. J. Nat. Prod. 50: 119-131, 1987.
6. Bancer, R.A., Hande, K.R. and Hamel, E.: The plant alkaloids. In: Chabner, B. (Ed): Pharmacologic Principles of Cancer Treatment, 2nd Ed., Philadelphia, W.B. Saunders Co. (in press).



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM-07104-12 LBP

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

L-Phenylalanine Mustard Cytotoxicity and Therapy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	D. T. Vistica	Pharmacologist	LBP, NCI
Others:	S. Ahmad	Visiting Fellow	LBP, NCI
	L. Okine	Visiting Fellow	LBP, NCI
	L. Nguyen	Biologist	LBP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.9	2.5	0.4

CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Murine L1210 leukemia cells resistant to the antineoplastic agent L-Phenylalanine Mustard have a 1.5-2.0 fold elevation in their cellular GSH and GSSG content. Cellular uptake of L-[<sup>14</sup>C(U)] cystine and its incorporation into GSH of the resistant tumor is correspondingly elevated. Synthesis of  $\gamma$ -glutamylcysteine, GSH and GSSG is elevated 1.5-2.0 fold in cell-free preparations of the resistant tumor. This increased synthesis of GSH is attributed to increased cellular content (1.6-fold) of  $\gamma$ -glutamylcysteine synthetase. GSH synthetase activity is equivalent in both drug sensitive and resistant cells. Investigation into the hydrolysis of selected peptides by cell-free preparations of both sensitive and resistant tumors suggest that Aminopeptidase M participates in the formation of L-cysteine from L-Cys-Gly. This is supported by the observation that these preparations readily degrade L-Leu-p-nitroanilide and L-Ala-L-Ala-L-Ala, known substrates for Aminopeptidase M, but not dipeptidase. The failure of the tumors to degrade Gly-D-Ala, a dipeptidase substrate, and the marked inhibition of L-Ala-Gly, L-Cys-Gly and L-Ala-L-Ala-L-Ala hydrolysis by bestatin further support a role for Aminopeptidase M in the generation of L-cysteine from L-Cys-Gly. These results suggest that the drug-resistant tumor cell has developed an efficient mechanism for maintenance of elevated GSH which involves both  $\gamma$  GT initiated catabolism of GSH to cysteine and its reutilization by  $\gamma$ -glutamylcysteine synthetase.



OBJECTIVES:

This project culminates several years of work whose primary objective has been to examine the relationship between resistance of tumor cells to alkylating agents and the tripeptide Glutathione (GSH). The work, described here, focuses on studies designed to determine the mechanism by which these drug-resistant tumor cells maintain their elevated GSH content.

METHODS EMPLOYED:Growth of L1210 and L1210/L-PAM Cells.

Cells were grown in RPMI 1630 medium supplemented with 16% heat-inactivated fetal bovine serum and 40 ug/ml gentamicin. Cells were subcultured every 2-3 days when the cell density reached  $1.0 \times 10^6$  cells/ml (Mid-logarithmic phase of growth).

Identification of GSH, GSSG and Biosynthetic Precursors of GSH.

Separation of amino acids utilized in the measurement of GSH biosynthesis from GSH and biosynthetic intermediates was accomplished according to the method of Griffith utilizing  $0.5 \times 7$  Cm columns of Dowex AG-IX-2 (acetate). The columns were calibrated prior to use with the respective amino acids, synthetic intermediates or products. Four hundred  $\mu$ l of cell-free supernatants, was applied to the column and the column was then washed with 4 ml of distilled water. This initial eluant contains L-cysteine, glycine, L-cystine and  $\gamma$ -aminobutyric acid. The column was then eluted consecutively with 2 ml of the following acetic solid solutions: (0.05 M, 0.1 M, 0.2 M, 0.4 M, 3 x 0.6 M, 0.8 M, 1.0 M, 1.2 M, 1.4 M, 1.6 M, 1.8 M, 2.0 M, 3.0 M, 4.0 M, 5.0 M). Glutathione is eluted with 0.8 to 1.2 M acetic acid while L-cysteinylglycine and  $\gamma$ -glutamylcysteine elute with 0.2 - 0.6 M and 3.0 - 5.0 M acetic acid respectively. GSSG and L-glutamic acid appear in the 1.4 - 1.6 M and 0.4 to 0.6 M acetic acid eluants respectively.

Biosynthesis of GSH, GSSG and  $\gamma$ -Glutamylcysteine by Cell-Free Lysates of L1210 and L1210/L-PAM.

Cell-free lysates of L1210 and L1210/L-PAM cells were prepared and dialyzed. The incubation mixture (1.0 ml) contained 0.1 TRIS • HCl buffer (pH 8.0),  $MgSO_4 \cdot 7H_2O$  (10 mM), KCl (10 mM), glycine (10 mM), L-cysteine • HCl (5.0 mM), ATP (10 mM), phosphocreatine (5.0 mM), creatine phosphokinase (20 units; 1 unit is that amount of enzyme which catalyzes the transfer of 1  $\mu$  mole of phosphate from phosphocreatine to ADP/minute at 30°C), L-[<sup>14</sup>C(U)] glutamic acid (5.0 mM), L- $\gamma$ -glutamyl (Ocarboxy) phenylhydrazide (0.25 mM) and protein (1.0 - 6.0 mg/ml).

Activity of  $\gamma$ -Glutamylcysteine Synthetase and GSH Synthetase in Cell-Free Lysates of L1210 and L1210/L-PAM.

These studies utilized protein prepared from Sephadex G-25 columns. The activity of  $\gamma$ -glutamylcysteine synthetase was determined by quantitation of  $\gamma$ -glutamylcysteine formation utilizing the incubation mixture described previously under Biosynthesis of GSH. Two modifications were employed: L-[<sup>35</sup>S] cysteine was utilized instead of L-[<sup>14</sup>C(U)] glutamic acid because



of its higher specific activity and glycine was omitted from the reaction mixture.  $\gamma$ -glutamylcysteine, which elutes with 3.0 - 5.0 M acetic acid, was measured by liquid scintillation spectrometry.

Glutathione synthetase was determined by substituting  $\gamma$ -glutamyl- $\alpha$  aminobutyric acid (5.0 mM) for L-cysteine and L-glutamate and using [ $^{14}$ C] glycine in the incubation mixture.  $\gamma$ -glutamyl- $\alpha$  aminobutyryl-glycine elutes with 0.4 to 0.8 M acetic acid.

#### Peptidase Activity in Cell-Free Lysates of L1210 and L1210/L-PAM.

The degradation of L-Leu-p-nitroanilide and selected peptides by cell-free lysates prepared on Sephadex G-25 columns as described previously was determined using the ninhydrin-cyanide assay. L-Leu-p-nitroanilide, L-Ala-Gly, L-Ala-L-Ala-L-Ala, Gly-D-Ala and L-Cys-Gly were used at final concentrations of 2.5 mM in a final volume of 800  $\mu$ l.

#### MAJOR FINDINGS:

1. The cellular transport of the GSH precursor amino acids glycine, L-cysteine, L-cystine and L-glutamic acid by L1210 and L1210/L-PAM cells is equivalent.
2. The synthesis of GSH, GSSG and T-glutamylcysteine is elevated 1.5 - 2.0 fold in the drug-resistant tumor cells.
3. The specific activity of T-glutamylcysteine synthetase is elevated 1.5 - 2.0 fold in the drug-resistant tumor cells while the activity of glutathione synthetase is equivalent.
4. The drug-resistant tumor cells have an enhanced ability to degrade selected peptides, including L-Cys-gly and this degradation is mediated by amino peptidase M.

#### PUBLICATIONS:

1. Ahmad, S., Mulberg, A., Aljian, J. and Vistica, D.T. Hepatic-mediated elevation and maintenance of metastatic tumor cell Glutathione. Biochem Pharmacol., 35: 1697-1701, 1986.
2. Vistica, D.T., Ahmad, S., Fuller, R. and Hill, J. Transport and cytotoxicity of amino acid nitrogen mustards: Implications for the design of more selective antitumor agents. Federation Proc., 45: 2449-2450, 1986.
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM-07179-02 LBP

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protein-protein and Protein-nucleotide Interactions in Microtubule Assembly

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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1.8	1.2	0.6

CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The rational development of new antineoplastic agents directed against tubulin, a protein critical for cell division, requires greater understanding of the interaction between the polypeptide subunits of tubulin, its two tightly bound guanine nucleotides, and microtubule-associated proteins. Copolymerization of tubulin-GDP and tubulin-GTP was studied in further detail, to determine the relative efficiencies with which the two species entered elongating microtubules and to define the minimum concentration of tubulin-GTP required to initiate microtubule assembly. The effects of nucleotides on the stability of microtubules continued to be examined, as were conditions to optimize the separation of  $\alpha$ -tubulin and  $\beta$ -tubulin on a preparative scale. The purification of a microtubule-associated protein which causes the formation of microtubule bundles continued to progress. Another microtubule-associated protein, which specifically degrades GDP to GMP, was observed and its purification initiated.



Microtubules, cellular organelles critical for cell division, are sensitive to a number of antineoplastic drugs. Their major constituent is an acidic protein known as tubulin, which consists of two different polypeptide chains and two molar equivalents of guanine nucleotide. Half this nucleotide (the exchangeable nucleotide) is in the form of either GTP or GDP. If GTP, it is hydrolyzed to GDP during microtubule assembly from tubulin and microtubule-associated proteins (MAPs -- minor, but essential, components of the microtubule). The remainder of the nucleotide exists only as GTP (the nonexchangeable nucleotide). This GTP is not altered during tubulin polymerization and can only be removed from tubulin by destroying the protein. Its function is unknown.

We have continued our studies on nucleotide interactions at the exchangeable site, with particular emphasis on reaction parameters previously shown to alter the ratio in which tubulin bearing GDP in the exchangeable site copolymerized with tubulin bearing GTP. These are all reaction components which increase the relative amounts of tubulin-GDP at the expense of tubulin-GTP--low GTP concentrations if the tubulin concentration is fixed; high tubulin concentrations if the GTP concentration is fixed; GDP in the reaction mixture from any source; low magnesium concentrations.

The tubulin-GDP-tubulin-GTP equilibrium was examined under multiple reaction conditions, as a function of tubulin, GDP, GTP, and  $Mg^{2+}$  concentrations, to determine the relative efficiencies with which the two species entered elongating microtubules and to define the minimum concentration of tubulin-GTP required for the initiation of microtubule assembly. The key parameter was found to be  $Mg^{2+}$ . Although the cation is required for the efficient binding of GTP (but not GDP) to tubulin, in its absence assembly could be initiated if as little as 20-25% of the tubulin was in the form of tubulin-GTP. In the absence of  $Mg^{2+}$ , however, tubulin-GDP was only half as efficient as tubulin-GTP in entering elongating microtubules. At the highest  $Mg^{2+}$  concentration examined (4 mM), 80% of the tubulin had to be in the form of tubulin-GTP for initiation, but tubulin-GDP was as efficient as tubulin-GTP in elongation.

We have continued to study the stability of microtubules as a function of their nucleotide content and environment. In particular, we are trying to determine whether stability is affected by the proportion of microtubule nucleotide which has resulted from hydrolysis of GTP to GDP as opposed to the proportion of GDP incorporated directly into the polymer. A related issue is the observation that microtubule integrity requires some GTP in the reaction mixture, for microtubules rapidly disintegrate if GTP is totally degraded by transfer of the terminal phosphate to fructose-6-phosphate by phosphofructokinase. Although we have established that nonexchangeable GTP is not destroyed in this reaction, we have yet to localize unambiguously the essential triphosphate.

For many years we have been attempting to reproducibly and preparatively separate the two subunits of tubulin. Although we had achieved significant separation by hydrophobic chromatography, reproducibility has been a problem. Continued efforts in the past year have not been successful. One goal of this project is reconstitution of activity from the separated subunits plus small ligands (i.e., GTP and/or GDP and  $Mg^{2+}$ ). Even in the absence of a totally successful separation of subunits, we have begun to search for conditions to reactivate denatured tubulin, using as a starting point previously determined reaction conditions in which tubulin is optimally stable.



We are continuing to devote a great deal of attention to MAPs. We are particularly interested in a MAP which causes the formation of microtubule bundles (distinct microtubules which aggregate laterally). The active component (termed MAP-TB) appears to be present in MAP preparations in extremely small amounts, although it is highly stable, and has proven more difficult to purify than anticipated. Despite DEAE-cellulose chromatography, ammonium sulfate fractionation, heat-treatment, hydroxyapatite chromatography, and HPLC chromatography (ion-exchange and gel filtration), the purest preparations remain disappointingly heterogeneous on polyacrylamide gel electrophoresis. Alternate ion exchange chromatography conditions have most recently been applied to this protein purification problem, and initial results have been promising. DEAE-cellulose chromatography at pH 11 and replacing CM-cellulose chromatography with "mono-S" chromatography have resulted in apparently purer preparations (but only on a small scale) than observed previously. The protein is unfortunately relatively labile at high pH.

In the course of preparing MAP-TB, because of the limited amounts obtained from microtubule protein, we have prepared large amounts of starting material. This includes most of the other MAPs present in the microtubules, and in processing this material we have developed superior methods of resolving some of the other MAPs components by DEAE-cellulose chromatography. In analyzing the properties of different MAPs fractions, we observed a peak of GDPase activity. It appears that this enzyme will not hydrolyze any other nucleotide, and generates only GMP and inorganic phosphate from GDP. We are attempting to purify this enzyme, because its presence in microtubule protein is probably significant--GDP is an inhibitor of microtubule assembly, and it would be desirable to be able to rapidly eliminate it. In addition, an enzyme capable of specifically eliminating GDP from reaction mixtures should be a valuable tool for studying tubulin-nucleotide interactions, in particular in the studies of microtubule stability described above.

#### PUBLICATIONS

1. Hamel, E. and Lin, C.M.: Dideoxyguanosine nucleotides and microtubule assembly. Ann. N.Y. Acad. Sci. 466: 635-638, 1986.
2. Hamel, E., Batra, J.K. and Lin, C.M.: Direct incorporation of guanosine 5'-diphosphate into microtubules without guanosine 5'-triphosphate hydrolysis. Biochemistry 25: 7054-7062, 1986.
3. Batra, J.K., Lin, C.M. and Hamel, E.: Nucleotide-interconversions in microtubule protein preparations, a significant complication for the accurate measurement of GTP hydrolysis in the presence of adenosine 5'-[ $\beta$ , $\gamma$ -imido]-triphosphate. Biochemistry, in press.
4. Lin, C.M. and Hamel, E.: Interrelationships of tubulin-GDP and tubulin-GTP in microtubule assembly. Biochemistry, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-CM-07181-02

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Antiretroviral Activity of Dideoxynucleosides

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(a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies on the cellular pharmacology of antiretroviral dideoxynucleosides have continued. We have observed that the anabolism of dideoxycytidine to its active form, ddCTP, is highly variable in both normal and neoplastic human cells, being sluggish in thoracic duct lymphocytes, energetic in monocytes and vigorous in ATH-8 T-lymphocytes. We also established that dideoxyadenosine, in contrast to dideoxycytidine, is catabolized extensively, both before but especially after its passage into cells. Since adenylylate deaminase accepts ddAMP as a substrate, and since dCF is not an effective inhibitor of this reaction, it is proposed that at least some of the catabolism of ddA is inaugurated at the level of its 5'-monophosphate, especially in the presence of dCF. As a consequence of the preponderant catabolism of ddA, only low levels of ddATP (50-100 nM) are detectable in cells treated with this nucleoside; these low levels must, nevertheless, be adequate to inhibit the reverse transcriptase of the HIV and block its replication.



## 1. Cellular Pharmacology of Dideoxyadenosine

The cellular pharmacology of the antiretroviral nucleoside, dideoxyadenosine (ddA) has been studied in cultured human lymphoblasts and a select number of other cell types. The compound is rapidly deaminated in serum-containing media ( $t_{1/2}$ : 2-5 hrs), and nearly instantaneously deaminated upon its passage across the cell membrane ( $t_{1/2} < 1$  min), so that dideoxyinosine (ddI) is the principal species seen in the cytoplasm. Deoxycoformycin (0.1  $\mu$ M) blocks both of these catabolic processes, and sharply reduces the accumulation of ddA-derived radioactivity in most cell lines. In three distinct adenosine-deaminase deficient lymphoblast cultures, entry of ddA was anomalous, being strictly equilibrative; by contrast, blasts containing normal ADA concentrate radioactivity attributable to ddI. We verified that this is not due to phosphorylation or ddI phosphorylation at early (< 15 min) exposure times.

By the use of enzyme-deficient mutants and site-selective antimetabolites, the intracellular fates of ddA/ddI have been dissected. In the presence of cytosolic phosphate, ddI is phosphorylyzed to hypoxanthine and dideoxyribose-1-phosphate (the latter probably rearranges chemically); the hypoxanthine is then anabolized to IMP, which is in turn converted to adenine and guanine ribonucleotides. This catabolic pathway accounts for >95% of the metabolized drug. The remaining ddI (ddA in the presence of dCF) is phosphorylated by several cytosolic and mitochondrial nucleoside kinases; through the agency of unidentified higher kinases. The resultant 5'-monophosphates are anabolized to the 5'-triphosphates, which probably function as the proximate antiretroviral species in their capacity as inhibitors of reverse transcriptase (RT). It is relevant to point out, however, that the  $K_i$  of ddITP for HIV RT is about one order of magnitude higher than that of ddATP whereas the drugs are equipotent in controlling HIV replication in the ATH-8 cell system of Broder.

For this reason, we sought to determine whether ddI (or ddIMP) can be converted to ddA (or ddAMP). The relevant deaminases ADA and adenylylate deaminase are effectively irreversible and so cannot be operative in this case; (adenylylate deaminase does accept ddAMP as substrate with moderate efficiency). Although purified adenylosuccinic acid synthetase does not appear to convert ddIMP to the dideoxy homologue of adenylosuccinate, exposure of human cells to [ $^3$ H] ddI does result in the appearance of a small pool of ddATP: thus the postulated interconversion must somehow be operative in the intact lymphoblast.

Lastly, because the anabolism of ddA to ddATP is such a quantitatively minor pathway, but of such great therapeutic importance, we are striving to stimulate it by pharmacologic manipulations.

Deoxycoformycin is the most obvious candidate for this role, but, as was mentioned earlier, this compound can markedly impede the interiorization of radioactivity derived from ddA in certain cell lines. Nevertheless, the appropriate concentration of dCF can enlarge the ddATP pool by 300% in CEM cells, although lesser expansion (140%) is the maximum seen in ATH-8 cells. Noteworthy is the finding that dCF does not enhance the antiviral activity of ddA. The natural nucleoside, deoxyadenosine (dA), also enhances the conversion of ddA to ddATP. In Molt/4 cells, this enhancement reaches 5-6 fold at 100  $\mu$ M dA and is dose-responsive from 5-100  $\mu$ M. At first it was believed that dA was acting through inhibition of adenosine deaminase, swamping that enzyme in its capacity as an



alternate substrate. However, when it was found that dA also equally enhanced the anabolism of ddI (to ddATP) this conjecture was abandoned. At present, it is hypothesized that dA after deamination to dI is acting at the level of purine nucleoside phosphorylase, preempting the active site of that enzyme in its capacity as a superior substrate, and so preserving ddI for an anabolic fate. Deoxyinosine will also augment the anabolism of ddA to ddATP, a result congruent with the foregoing hypothesis. Similarly, the ability of dA to enhance the antiretroviral potency of ddA, while under investigation, has so far not been established unequivocally. Nevertheless, in view of the quantitatively minor nature of its anabolism, it is obvious that therapeutic strategies are needed to promote the conversion of ddA to ddATP.

2. Measurement of the Chemical Stability of ddATP and Optimization of Techniques for Extracting that Nucleotide from Biological Samples.

Although the instability of ddA has been reported in the chemical literature, not all biochemists have taken the acid lability of this nucleoside into account. The same susceptibility to acid-catalyzed depurination which characterizes ddA should also obtain with its nucleotides. To verify this lability, we have measured the breakdown of ddATP at pH 1 (gastric pH in humans) and additional pH's, including neutrality, with the results presented in Table 1. It can be seen that depurination of both ddA and ddATP is nearly instantaneous at pH 1 but slows remarkably as neutrality is approached. This result, of course, precludes the use of customary acidic extractants (TCA or PCA) in the measurement of the metabolites of this and all other purine dideoxynucleosides, namely ddI and ddG. We have therefore compared a variety of other neutral extraction techniques, assessing by HPLC the recovery with them of ddATP added as a spike to frozen pelleted cells. While no technique so far deployed has yielded > 90% recoveries of exogenous ddATP, 60% methanol extraction followed by immediate boiling for 2 minutes in a water bath has proven to yield the most acceptable (60%) and the most reproducible recoveries. Thus, although we have in the past year used and reported other extraction techniques (boiling distilled water, cold 60% methanol) we would now propose the aforementioned two-step technique for the extraction of ddATP from cultured cells. At present however, there is no useful technique for the extraction of ddA metabolites from specimens such as solid tissues and tissue biopsies.

Table 1. Chemical stability of ddA and ddATP at various pH values.

pH	ddA	$t_{1/2}$ (min)	ddATP
1	0.43	<1.0	
2	5.30	12.0	
4	ND	94.0	
6	ND	>24 hr	
7.4	>24 hr		>24 hr

Legend: Sodium phosphate buffers (0.1 M) of the indicated pH were prepared by



standard methods. Initial ddA concentrations were 42.  $\mu$ M and ddATP concentrations were 400  $\mu$ M. Appearance of adenine and disappearances of ddA were measured by reverse-phase HPLC; disappearance of ddATP was measured by ion-exchanged HPLC. Values of  $t_{1/2}$  greater than 1 hr were estimated by extrapolation. ND: Not determined.

3. Preparation of Oligonucleotides Capped with Dideoxynucleotides-5-mono-phosphates; Enzymologic and Antiretroviral Properties.

An examination of the genome of a typical HIV isolate shows that there are over 25 oligo (5-6) adenylate sequences present therein. By contrast, there are only 7 oligo G and 5 oligo T sequences in the genome of this virus. It therefore seemed advisable to capitalize on the abundance of the adenine containing regions by designing small penta and hexaoligonucleotides sequences, capped, if possible, by residues with chain-terminating potential. The 2',3'-dideoxynucleosides appear to offer this capability. Toward this end, then, oligothymidylic acids, consisting of 6-12 residues were terminated enzymatically with several 2',3'-dideoxynucleoside monophosphates, using terminal deoxynucleoside nucleotidyl-transferase as the capping enzyme. The resulting oligomers were purified by HPLC sizing chromatography, desalting, and tested in two ways: as inhibitors of reverse transcriptase and as antiretroviral agents in the ATH-8 system. Preliminary results indicate that oligomers of thymidylic acid capped with ddA inhibit reverse transcriptase from HIV with an IC<sub>50</sub> of 10  $\mu$ M - a value approximately twenty times that of free ddATP. In the T-cell system, the capped oligomer has so far proven to be inferior to ddA producing only modest (30%) decreases in infectivity, at concentrations at which the parent nucleoside yields complete protection. One obvious reason for this loss of potency was provided by studies of the uptake of radiolabeled capped oligomers: cellular penetration of drug-derived radioactivity is slow, minor in quantity, and apparently associated with cleavage products.

4. Studies with Dideoxycytidine.

In last year's annual report, the metabolism of dideoxycytidine (ddC) in cultured murine cells was recapitulated in detail. During the present year we have extended those studies to a large panel of normal and neoplastic human cells, including T and B lymphoblasts, thoracic duct lymphocytes from a normal donor, and fresh monocytes obtained by centrifugal elutriation. In all the lines so far examined, the metabolism of ddC has conformed in a qualitative way to that seen previously in P388 and L1210 lymphoblasts: that is, the drug is anabolized to the level of the 5'-mono, di and triphosphate. There is seen, in addition, a fourth metabolite, provisionally identified in last year's report as ddCDP choline. The quantitative aspects of the metabolism of ddC in normal human cells present several interesting variations: thus in thoracic duct lymphocytes, phosphorylation is remarkably scanty, whereas, in elutriated monocytes it is surprisingly energetic. Metabolism in immortalized B-lymphocytes falls between these extremes (Table 2).

The enzymological bases for these variations have not been established, but it is reasonable to implicate the specific activity of deoxycytidine kinase in their causation, inasmuch as our previous studies established that enzyme as the rate-limiting step in the anabolism of the drug.



In the present year, too, further steps were taken to verify the composition of the phosphodiester-sensitive metabolite of ddC, which had been tentatively identified as ddCDP choline. Using a purified preparation of CTP-phosphorylcholine phosphotransferase and either radiolabeled [<sup>3</sup>H]ddCTP or <sup>14</sup>C-phosphorylcholine, we have succeeded in preparing a molecule whose chromatographic properties and susceptibility to enzymic cleavage are identical to the diester extracted from ATH-8 and other cells. While attempts to prepare ddCDP choline chemically by the method of Kennedy have so far failed, a new route to its synthesis is being actively explored by DCT. If obtained, this compound will be tested against HIV in ATH-8 cells in order to ascertain whether it offers any therapeutic advantages over ddC on the basis of its enhanced lipophilicity.

Table 2. Anabolism of ddCyd in a panel of human cells.

Cell line	Cell type	pmoles/10 <sup>6</sup> cells				
		ddCyd	ddCMP	ddCDPCh	ddCDP	ddCTP
T-Hem (C)	T-lymphoblasts	0.3	0.3	0.1	0.6	0.6
B-Hem (C)	B-lymphocytes	1.7	0.4	0.3	0.7	0.8
VDSO (C)	B-lymphocytes (EBV-infected)	1.1	0.6	0.2	0.8	0.3
THP-1 (C)	Monocytes	0.6	0.3	0.1	0.1	0.3
V-937 (C)	Monocytes	0.6	7.9	2.6	4.8	2.2
H-9 (C)	T-lymphocytes	0.9	3.5	1.4	1.5	1.6
Thor-1 (F)	Thoracic duct lymphocytes	10.0	<0.01	0.02	0.04	0.03
Mono-1 (F)	Monocytes	0.02	0.21	0.07	0.24	1.50
PHA (F)	PHA-stimulated lymphocytes	1.71	0.32	0.22	0.08	0.32

Legend: After incubation with [<sup>3</sup>H]ddCyd (0.5  $\mu$ M) for 24 hr at 37° C, cells were extracted with 10% TCA and anabolites were resolved by HPLC. Monocytes were obtained from a normal donor by centrifugal elutriation. C: Propagated in culture; F: fresh isolates.

##### 5. Further Attempts to Enhance the Anabolism of ddC.

Recently we reported that high concentrations of thymidine and several, but not all pyrimidine antimetabolites were capable of stimulating the phosphorylation of ddC to ddCTP by factors of from 3 to 7 fold. This enhancement was believed to result from a depletion of dCTP pools. This in turn relieved the inhibition of deoxycytidine kinase produced by the natural deoxyribonucleotide, enabling a



more energetic phosphorylation of its unnatural counterpart, ddC. In the present year we examined the capacity of a select number of other putative modulators to achieve an analogous enhancement. So far no modulators comparable in potency to thymidine have been identified but we have observed that the deoxycytidine/deoxycytidylate deaminase inhibitor 2'-deoxytetrahydouridine (dTNU) (used in an attempt to spare ddCMP from catabolism) produces a very large (20-fold) expansion of dCTP pools. Since, as was mentioned, dCTP is a powerful end-product inhibitor of deoxycytidine kinase, it is clear that dTNU should not be used in combination with ddC as a therapeutic strategy. It is relevant to note that congenital deficiencies of deoxycytidylate deaminase are also accompanied by large expansions of the dCTP pools so that treatment with dTNU would appear to provide a pharmacologic paradigm of the natural lesion.

## 6. Studies with 2',3'-Dideoxyuridine and its 5'-Triphosphate.

Although dideoxyuridine (ddU) itself is devoid of antiretroviral activity ( $EC_{50} > 500 \mu M$ ), this inefficacy might be attributed to a failure of the appropriate cellular enzymes to anabolize the compound to the level of the 5'-triphosphate as a consequence of the prohibitive constraints imposed by the distinctly unnatural 2',3'-dideoxy conformation of its "ribose" moiety. Indeed when ATH-8 lymphoblasts are exposed to 1  $\mu M$  ddU for 24 hours, the levels of the 5'-triphosphate of this agent measurable by HPLC are ~ 200 times lower than those achieved by the congeneric pyrimidine nucleoside ddC (5 nM vs 1  $\mu M$ ). Nevertheless, because the mispairing purine dideoxynucleoside-5'-triphosphate ddITP can inhibit reverse transcriptase (albeit with 10x lower potency than either ddATP or ddGTP), we felt that it would be advisable in the interest of systematicity to synthesize and test a pyrimidine dideoxyribonucleoside-5'-triphosphate with an analogous capacity to mispair: ddUTP was the most obvious such choice. Toward this end, commercially available ddCTP, (20  $\mu$ moles of the sodium salt) was incubated overnight at 37° in a saturated aqueous solution of sodium metabisulfite at pH 6.5. The bisulfite adduct was hydrolyzed to ddUTP by ascending paper chromatography at pH 8.9 (EtOH, 70 parts; 1 M NH<sub>4</sub> acetate 30 parts; NH<sub>4</sub>OH 1 part) and repurified by a second ascending chromatography at pH 3 (ethanol 55 parts; formic acid 20 parts; H<sub>2</sub>O 25 parts). The resultant preparation (20% yield) was homogeneous on HPLC. Surprisingly, when tested against the reverse transcriptase from HIV, ddUTP proved to be a preeminently potent inhibitor of the enzyme (Table 3); against AMV RT, it was 40 times less potent. Thus it appears that the catalytic centers of these two polymerases are strikingly different in their susceptibility to this dideoxypyrimidine-5'-triphosphate. Since the anabolism of ddU is so feeble in mammalian lymphoblasts, it is planned to introduce ddUTP into HIV-infected cells through the agency of liposomes prepared in collaboration with Dr. R. Parker.



Table 3. Selective inhibitory effect of ddUTP on HIV and AMV reverse transcriptases in two template-primer systems

Template-primer: substrate	IC <sub>50</sub> (μM) vs. RT	
	AMV	HIV
PolyIdC <sub>18</sub> :dCTP	8.2	15.9
PolyAdT <sub>15</sub> :dTTP	4.0	0.1

Legend: Assays were performed using FPLC-purified AMV RT (2.0 U) or purified HIV RT (0.9 U); polyIdC<sub>18</sub> (1.25 μg/25 μl), [<sup>3</sup>H]dCTP (1.5 μM) (specific activity 24 Ci/mmol); polyAdT<sub>15</sub> (1.25 μg/25 μl), [<sup>3</sup>H]dTTP (1.25 μM) (specific activity 67 Ci/mmol). Products were separated by ascending paper chromatography (EtOH:formic acid:H<sub>2</sub>O: 55:20:25). Polymerized products remain as condensed spots at the origin in this system. The ddUTP used in these studies was verified to be free (<1%) of ddCTP by ion-exchange HPLC.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06140-11 LMPH

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Histone Biosynthesis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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0.6

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0.3

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 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our objective is to elucidate the factors involved in the close co-ordination of the synthesis of histone protein and DNA. Recently we published a model which suggested that inhibition of protein synthesis led to the inhibition of DNA synthesis by the depletion of histone from the cytoplasmic pool. This model was simpler than others in that the resulting stabilization of histone mRNA could be viewed as part of the same process that led to its destabilization when DNA synthesis was inhibited.

We have developed methodology which allows one to study the flux of histone through the cytoplasm and alterations in that rate of flux when protein or DNA synthesis has been inhibited. These methods can be applied to cells in different states of growth (G1, G0, and S).

Results currently being prepared for publication show that when protein synthesis is inhibited, the rate of DNA synthesis and the level of cytoplasmic histone both fall in a biphasic manner with similar kinetics. These results are completely consistent with our proposed model that DNA synthesis can be physiologically limited directly by availability of histone.

When DNA synthesis is inhibited, the histone level in the cytoplasmic pool is elevated, with H4 and H3 being elevated more than H2B and H2A. We have also been able to demonstrate that H2B and H2A from prelabeled nuclei can be found in the cytoplasm while H4 and H3 are not. This result suggests that the mechanism is operating in G1 cells as in S cells with inhibited DNA synthesis. Experiments with G0 cells are in progress.



Project DescriptionIntroduction:

We have found that when dividing cells cease division and become quiescent, they continue to synthesize histones at a reduced but significant rate. The pattern of synthesis of variant histones in quiescent cells differs from that in S-phase; therefore, this synthesis cannot be attributed to the presence of S-phase cells in the culture.

In CHO cells where the pattern of histone synthesis has been examined during the cell cycle, reduced but significant synthesis has been found in both G2 and G1 (Wu and Bonner, Cell 27: 321-330, 1981). This basal pattern differs from both the S-phase and the quiescent patterns. These results strongly suggest that the quiescent state is not merely an extended G1, but is a discrete state or cycle.

Objectives:

- 1) The development of greater understanding of the molecular mechanisms regulating chromatin biosynthesis and metabolism during the cellular states of proliferation and nonproliferation.
- 2) The characterization of chromatin or cellular components that may be involved in these regulatory mechanisms. At present the cytoplasmic histone pool is receiving most of our attention.

Methods:

- (1) Discontinuous electrophoretic separation of histones including direct loading of histone extracts and two dimensional electrophoresis. (Methods developed in this laboratory).
- (2) Peptide analyses on acrylamide gels to determine the relationship of proteins to each other. (Method developed in this laboratory).
- (3) Synchronization of cell lines, particularly human HeLa cells and Chinese hamster ovary cells for studies on cell cycle.
- (4) Maintenance of cells and nuclei in viable non dividing states using modified and defined media.
- (5) Isolation and analysis of mRNA from different parts of the cell cycle or from quiescent cells. Cell free translation of mRNA.

Major Findings and Accomplishments:1. Effects of Inhibitors of DNA and Protein Synthesis on G1, Quiescent, and S-Phase Histone Synthesis to DNA Synthesis

Many studies have shown that inhibition of DNA synthesis immediately leads to a similar inhibition of histone synthesis even though total protein synthesis is



not significantly inhibited. Our studies with hydroxyurea, a classical inhibitor of DNA synthesis, show that basal and S phase histone synthesis are inhibited to different extents when DNA synthesis is inhibited. We have continued these studies with a variety of inhibitors of DNA synthesis. Those agents which inhibit DNA synthesis only, give the same results as hydroxyurea. However other agents which also inhibit protein syntheses do not selectively inhibit the synthesis of particular histone variants but change the histone synthesis pattern in other ways. The crosslinking antineoplastic agents are in this class. It has previously been reported that inhibition of protein synthesis blocks the specific effect of DNA synthesis inhibitors on histone synthesis. We have published a model that offers an explanation for these interactions.

## 2. Development of Methodology to Study Histone Pools

In this model, the amount of histone in the cytoplasm is proposed to be a the central control element in the regulation of histone and DNA synthesis. However, the histone pool has not been studied in any systematic way because of several technical difficulties. These include the small size of the pool, the problems of purifying histone proteins from the cytoplasm, and the problem of distinguishing cytoplasmic histone from contaminating chromatin histone. Those few studies of histone pools have examined the labeling kinetics of some of the histone proteins rather than their mass.

We have adapted our methodology for the analysis of histone variants in chromatin to the analysis of histones in the cytoplasm and have overcome most of these problems. Newly synthesized H4 in the cytoplasm is doubly modified, by an acetate and a phosphate. When cytoplasm is prepared by gentle lysis of cells with a nonionic detergent, then extracted with HCl and the extract freeze dried for electrophoresis on AUT-AUC gels, the pattern of cytoplasmic histone shows doubly modified H4 as well as the absence of ubiquitin adducts of the H2A's. Thus cytoplasmic histone can be analyzed with little or no contamination from nuclear histone.

We plan to use this newly developed method to study several aspects of the histone pool. The first set of questions concern the relationship of the pool histone to chromatin histone during normal S-phase. Do histones flux in one direction only from polyribosomes, through the pool to chromatin, or do histones flux out of chromatin into the pool? Are histones in the pool degraded? The second set of questions concern how the histones in the pool react to the inhibition of DNA and/or protein synthesis. Do the levels of histones in the pool change in such a way as to be consistent with auto-regulation. The third set of questions concern the level and flux of histone in G1 and G0 as well as in S, and the transition between these states.

## 3. Investigations of the Cytoplasmic Histone Pool

Histone protein synthesis and DNA synthesis are closely co-ordinated; inhibition of one leads to inhibition of the other. However, when DNA synthesis is inhibited, histone mRNA levels fall and when protein synthesis is inhibited, histone mRNA levels rise. Recently this Section published a model which suggested that inhibition of protein synthesis led to the inhibition of DNA



synthesis by the depletion of histone from the cytoplasmic pool. This model was simpler than others in that the resulting stabilization of histone mRNA could be viewed as the same process that led to its destabilization when DNA synthesis was inhibited.

Methodology has been developed which allows one to study the flux of histone through the cytoplasm and alterations in that rate of flux when protein or DNA synthesis has been inhibited. These methods can be applied to cells in different states of growth (G1, G0, and S).

Results currently being prepared for publication show that when protein synthesis is inhibited, the rate of DNA synthesis falls in a biphasic manner. The initial fall has a half time of 2 min. When the rate of DNA synthesis has fallen to 25% of the initial rate, the rate then falls more slowly with a half time of 25 min. The rate of histone depletion from the pool is also biphasic. The initial decrease in the rate of DNA synthesis coincides with the initial rate of histone depletion from the cytoplasmic pool. When the histone pool is depleted to about 50%, the rate of depletion slows to a rate similar to the slower decrease in the rate of DNA synthesis. These results are completely consistent with our model that DNA synthesis can be physiologically limited directed by the availability of histone.

When DNA synthesis is inhibited, the histone level in the cytoplasmic pool is elevated, with H4 and H3 being elevated more than H2B and H2A. The latter two histones continue to be incorporated into chromatin for sometime in the absence of DNA synthesis. We have also been able to demonstrate that H2B and H2A from prelabeled nuclei can be found in the cytoplasm while H4 and H3 are not. This result suggests that H2B and H2A can exchange between the nucleus and cytoplasm in both directions.

Further experiments with G1 cultures have shown that H4 and H3 are elevated in the cytoplasm, while H2B and H2A are not detectable. This finding suggests that the same mechanism is operating in G1 cells as is in S cells with inhibited DNA synthesis. Experiments with G0 cells are in progress.

Manuscripts for these findings are currently in preparation.

Significance to Biomedical Research and Program of the Institute:

Cancer at one level is the inappropriate multiplication of cells. Our findings during the last few years have suggested that analysis of histone variant synthesis and the histone variant genes may yield some insight into the relationship of different cell states in normal and neoplastic cells.

Proposed Course:

1. To characterize the histone pool and to set the predictions of the published model as to the behavior of the pool.
2. To develop methodology to study the selective sensitivity of histone mRNA.



3. To investigate the biochemical aspects of the exchange of H2B and H2A from nuclei to cytoplasm.

Publications:

Two in preparation.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06150-06 LMPH

## PERIOD COVERED

October 1, 1986 to September 31, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protein-Associated DNA Strand Breaks as an Indicator of Topoisomerase II Inhibition

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Yves Pommier	Visiting Associate	LMPH NCI
Others:	Donna Kerrigan	Chemist	LMPH NCI
	Joseph Covey	Senior Staff Fellow	LMPH NCI
	Kurt W. Kohn	Lab. Chief	LMPH NCI

## COOPERATING UNITS (if any)

Jacqueline Whang-Peng, Sr. Investigator, MB, NCI; Chien-Song Kao-Shan, Cytogeneticist, MB, NCI; Biranda Sinha, Sr. Investigator, LCP, NCI

## LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT, NCI

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two families of anticancer drugs have been shown to be topoisomerase II inhibitors: DNA intercalators, such as amsacrine [m-AMSA], adriamycin [ADR] and ellipticinium [2-Me-9-OH-E-], and demethylepipodophyllotoxins, such as etoposide (VP-16) and teniposide (VM-26). Topoisomerase II (topo II) is a ubiquitous enzyme involved in chromatin structure regulation by controlling both DNA topology (supercoiling, catenation, etc.) and chromatin loop attachment to the nuclear matrix. Topo II is important in DNA replication, transcription and chromatin packaging. Drug inhibition stabilizes topo II-DNA intermediates, which can be detected as protein-associated DNA strand breaks. We have shown previously that rapidly proliferating and S-phase cells are more drug sensitive than quiescent cells. Now, we have established that newly replicated DNA forms more drug-induced topo II-mediated DNA breaks than bulk DNA and that replicating DNA strands become linked to topo II after they have reached replicon size. We have also investigated the relationship between drug-induced topo II-mediated DNA breaks and cytotoxicity. A clear correlation is observed within drugs of a single chemical group (acridines, demethylepipodophyllotoxins). Finally, we are trying to identify the biochemical topoisomerase modifications that account for drug resistance in cells resistant to topoisomerase II inhibitors.



Objectives:

1. Study the role of DNA topoisomerase II in DNA replication and its location within replicons.
2. Study the relationship between drug effects on topoisomerase II and cytotoxicity, and the cellular events leading to cell death upon exposure to antitumor topoisomerase II inhibitors.
3. Isolate topoisomerases from resistant cells and determine the relationship between nuclear and plasma membrane modifications in these pleiotropic resistant cells.

Methods:

1. Standard cell culture, nuclei isolation and synchronization methods.
2. Alkaline elution methodology to determine drug-induced topoisomerase II-mediated DNA strand breaks and DNA-protein crosslinks.
3. Alkaline sucrose sedimentation.
4. Clonogenic assays to measure drug-induced cytotoxicity.
5. DNA isolation procedures, restriction enzyme digestion, agarose gel electrophoresis, Southern transfer, and hybridization with various genomic probes.

Major Findings:

1. Relationship Between Topoisomerase II and DNA Replication.

Mouse NIH 3T3 cells stop proliferation when they reach a certain density on the plate at 1% serum concentration. The same cells can be initiated to proliferate synchronously by replating them in 10% serum at lower cell density. We have shown previously that m-AMSA- and VP-16-induced topoisomerase II-mediated DNA damage were nearly nonexistent in growth arrested cells and maximum during DNA synthesis. Salt extracts of cell nuclei showed also highest topoisomerase II activity during cell proliferation and DNA replication. The relationship between topoisomerase II and DNA replication was further investigated in S-phase synchronized 3T3 cells [(t23 after replating quiescent cells in 10% serum)]. Two additional observations suggest now that topoisomerase II could play a role in DNA replication. 1) Both m-AMSA and VP-16 inhibited DNA synthesis within a few minutes after drug exposure. This inhibition occurred as quickly as topoisomerase II-mediated DNA breaks, but was prolonged (more than 60 min) after drug removal, while DNA breaks were reversible. 2) Approximately twice as many topoisomerase II-mediated DNA strand breaks were produced in newly replicated DNA as compared to uniformly labeled template DNA. The location of drug-induced topoisomerase II-DNA complexes with respect to the replication fork was determined by pH-step elution. From these experiments, it appears that drug-induced topoisomerase II-DNA binding does not occur at the replication fork on the replicating DNA strand, and that it is not inhibited by DNA replication fork movement blockade by aphidicolin. On the



contrary, newly replicated DNA strands appear to become linked to topoisomerase II after they have reached a certain length. Alkaline sucrose gradient experiments are in progress to determine this critical length with respect to replicon size.

## 2. Relationship Between Drug-Induced Topoisomerase II-mediated DNA Damage and Cytotoxicity. Processing of Topoisomerase II-mediated DNA Damage into Lethal Cellular Events.

Several experimental approaches have been undertaken to relate drug-induced topoisomerase II inhibition by drugs and cytotoxicity. One of them has been to determine whether cells that had been made resistant to topoisomerase II inhibitors would form drug-induced topoisomerase II DNA damage. Several laboratories, including ours (Pommier et al., Cancer Res., 1986) have taken this approach and obtained similar results. Resistant cells form markedly less topoisomerase II-mediated DNA breaks upon exposure to inhibitors. A second approach has been to correlate drug-induced DNA damage and cytotoxicity for a variety of compounds within a single cell line. Previous studies did not show a good correlation when compounds of various chemical classes were compared. This may be due to the fact that the location of drug-induced topoisomerase II-mediated DNA breaks differs for each class of inhibitor (see section CM-06161-06). For this reason, we have limited our comparison to drugs which gave similar DNA cleavage distribution in purified systems. Two classes of topoisomerase II inhibitors were studied in mouse leukemia L1210 cells, m-AMSA and VP-16 derivatives (Covey et al., Cancer Res., in press; Kerrigan et al.). In each chemical class, drugs varied widely in their potency to induce topoisomerase II-mediated DNA damage: VM-26 was approximately 10-fold more potent than VP-16, 9-aminoacridine was not active and some 9-aminoacridines were more potent than m-AMSA. Similar differences were observed for drug-induced cytotoxicities. Single regression lines were obtained by plotting drug-induced topoisomerase II-mediated DNA damage against cytotoxicity for 9-aminoacridine derivatives and for demethylepipodophyllotoxins. In addition, in the case of VP-16 and VM-26, the regression lines were similar in three different cell lines, mouse leukemia L1210, human colon carcinoma HT-29, and human embryonic fibroblast VA-13 cells, despite the fact that these three cell lines had very different drug sensitivities. Therefore, there is a good relationship between drug-induced topoisomerase II-mediated DNA strand breaks and drug-induced cytotoxicities within a single chemical class of topoisomerase II inhibitors. However, the reversibility of topoisomerase II-mediated DNA strand breaks in cells that will die implies that these breaks may lead to other cellular damages that are not reversible upon drug removal. We have previously postulated that genomic rearrangements, such as sister chromatid exchanges (SCE) and mutations could occur in the presence of drug-induced topoisomerase II-mediated DNA strand breaks (Pommier et al., Cancer Res., 1985). Thus, drug-induced topoisomerase II-mediated DNA strand breaks could be the initial cellular damage involved in the antitumor activity of topoisomerase II inhibitors. Secondary effect, such as chromosomal and genetic rearrangements could be the lethal damage that actually kills the cell.

## 3. Topoisomerase II Modifications in Drug-resistant Cells.

We have shown previously that a line of Chinese hamster cells that had been



made resistant to 9-OH-ellipticine was cross-resistant to other topoisomerase II inhibitors to which the cells had not been selected with, and that their resistance phenotype belonged to the pleiotropic drug resistant cell family. We have shown also (Pommier et al., Cancer Res., 1986) that these cells had nuclear modifications such that their topoisomerases II were only slightly decreased in copy number and their topoisomerases I were possibly mutated. We have been developing new topoisomerase purification methods by using FPLC techniques. FPLC methodology allows faster and more reproducible isolations. 0.35 M salt extracts are first made from cell nuclei. These extracts are then fractionated by Mono Q chromatography (anion exchange), which yields almost pure topoisomerases in one step. However, topoisomerases II copurify usually with topoisomerases I, and a second purification step is required to separate the two enzymes. We have been using successfully glycerol gradient centrifugation for that purpose. More recently, we have started using FPLC gel filtration (Superose) instead of glycerol gradient centrifugation in order to faster separate faster topoisomerases II & I and to isolate the mutated topoisomerases present in resistant cells. In the same line of research, pleiotropic adriamycin resistant cells from a breast cancer cell line (MCF7/ADR) have been studied. The question was whether pleiotropic resistant cells would have topoisomerase modifications as well. MCF7/ADR cells turned out to be cross-resistant to etoposide and this resistance could not be accounted for by drug uptake reduction. In addition, MCF7/ADR cells did not produce etoposide-induced DNA breaks upon drug exposure. Ongoing studies are investigating whether isolated nuclei from MCF7/ADR cells are also resistant to the DNA breaking effect (topoisomerase II-mediated) of etoposide. If it were the case, other pleiotropic resistant cell lines could be investigated in order to determine whether topoisomerases modifications belong to the common phenotype of pleiotropic drug resistant cells.

Proposed Course:

1. Determine whether DNA replication inhibition or synchronization by aphidicolin alters the cytotoxicity and the DNA breaks induced by topoisomerase II inhibitors.
2. Investigate the resistance mechanism(s) of human breast cancer cells resistant to adriamycin and VP-16 (MCF7/ADR).
3. Isolate DNA topoisomerases from sensitive and resistant cell lines by FPLC and determine their chemical, enzymic, and drug sensitivity characteristics. A long term project is to isolate the gene(s) of the modified topoisomerases for resistant cells.
4. Determine the genomic localization of drug-induced topoisomerase II-mediated DNA strand breaks in the dihydrofolate reductase and metallothionein genes and compare the DNA cleavage intensities and patterns in normal and gene amplified cells.

Publications:

1. Pommier, Y., Kerrigan, D., Schwartz, R., Swack, J., and McCurdy, A.: Altered topoisomerase II activity in Chinese hamster cells resistant to



topoisomerase II inhibitors. Cancer Res. 46: 3075-3081, 1986.

2. Markovits, J., Pommier, Y., Kerrigan, D., Covey, J.M., Tilchen, E.J., and Kohn, K.W.: Topoisomerase II-mediated DNA breaks and cytotoxicity in relation to cell proliferation and the cell cycle in NIH 3T3 fibroblasts and L1210 leukemia cells. Cancer Res. 47: 2050-2055, 1987.

3. Pommier, Y., Kerrigan, D., and Kohn, K.W.: Topoisomerase alterations associated with drug resistance in a line of Chinese hamster cells. Proceedings of the First Conference on Topoisomerases in Cancer Chemotherapy, Natl. Cancer Inst. Monogr., in press.

4. Kerrigan, D., Pommier, Y., and Kohn, K.W.: Protein-linked DNA strand breaks produced by etoposide (VP-16) and teniposide (VM-26) in mouse L1210, and human VA-13 and HT-29 cell lines. Relationship to cytotoxicity. Proceedings of the First Conference on Topoisomerase in Cancer Chemotherapy, Natl. Cancer Inst. Monogr., in press.

5. Kohn, K.W., Pommier, Y., Kerrigan, D., Markovits, J., and Covey, J.: Topoisomerase II as a target of anticancer drug action in mammalian cells. Proceedings of the First Conference on Topoisomerases in Cancer Chemotherapy, Natl. Cancer Inst. Monogr., in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CM 06161-04 LMPH

PERIOD COVERED

October 1, 1986 to September 31, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Topoisomerase II as Target of Action of Anticancer Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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COOPERATING UNITS (if any)

Cancer Research Laboratory, University of Auckland, New-Zealand (Dr. Baguley), Chemistry and Life Sciences Division, Research Triangle Institute, Research Triangle Park, NC 27709 (Dr. M. Wall).

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

DNA topoisomerases II (topo II) cause the DNA breaks and DNA-protein crosslinks observed upon exposure of mammalian cells to antitumor DNA intercalators (adriamycin, amsacrine, ellipticine) and demethylepidopodophyllotoxins (VP-16, VM-26). Topo II can be purified from mouse leukemia L1210 and Chinese hamster lung fibroblasts (DC3F and DC3F/9-OHE) by FPLC. We have shown previously that drug-induced DNA breaks and DNA-protein crosslinks can be reproduced in purified systems. A method has been developed which allows the mapping of drug-induced topo II-mediated DNA break sites by using [<sup>32</sup>P]-end labeled DNAs, agarose or sequencing gels, autoradiography and computer analysis. Drug-induced DNA cleavage sites were mapped within SV40 DNA. Drugs appeared to enhance DNA cleavage at sites that were already cut by topo II alone. Each chemical class of topo II inhibitors exhibited a selective enhancement pattern. In the case of amsacrine derivatives, DNA intercalation was not correlated with topo II inhibition potency and DNA sequence selectivity of binding did not influence the cleavage pattern. Thus the differential enhancement of DNA cleavage sites by topo II inhibitors may explain differential drug cytotoxicity. A second type of studies was to investigate the effects of DNA groove binders upon topo II. Polyamines (spermine, spermidine) were shown to modulate topo II DNA catalytic activities and drug-induced topo II breaks. DNA binding of the minor groove binder, distamycin was shown to change the distribution of drug-induced topo II-mediated DNA breaks. Finally, drug effects upon purified topo I were also studied. DNA intercalators appear to inhibit topo I and this effect may contribute to the antitumor activity of these drugs.



Objectives:

1. Improve the purification procedures of topoisomerase II from mammalian cells in culture.
2. Study the mechanism(s) by which antitumor drugs inhibit purified DNA topoisomerase II and how this inhibition relates to DNA binding.
3. Determine the genomic and DNA sequence location of drug-induced topo-isomerase II-mediated DNA breaks in purified SV40 DNA.
4. Study the effects of minor groove DNA binding drugs (polyamines, distamycin A) upon topoisomerase II.
5. Analyze topoisomerase I inhibition by DNA intercalators and camptothecin analogs.

Methods:

1. Purification of DNA topoisomerases from mammalian cells in culture by FPLC (anion exchange chromatography, superose gel filtration).
2. Agarose gel electrophoresis (+ chloroquine) to separate supercoiled, relaxed, cleaved, nicked and catenated DNA molecules. Filter binding assay to detect topoisomerase-DNA crosslinks.
3. [<sup>32</sup>P]-end labeling of SV40 or pBR322 DNAs, followed by autoradiography of agarose gels and sequencing gels to locate topoisomerase-induced DNA breaks.
4. SDS-PAGE electrophoresis and immunoblotting to visualize and quantify DNA topoisomerases.

Major Findings:1. Purification of DNA Topoisomerases by FPLC.

Our previous topoisomerases purification methods (gel filtration, DNA-cellulose chromatography, glycerol gradient centrifugation) allowed enzyme isolation in a week. FPLC is a computerized, reproducible and fast method for protein isolation. Topoisomerases can be purified in 3-4 days. The present steps of our purification protocol are: 1) Nuclei isolation from  $10^8$ - $10^9$  cells ( $\sim 10$  g); 2) extraction of the nuclear proteins soluble in 0.35 M NaCl; 3) clarification of the salt extract by glass fiber filter filtration; 4) gross fractionation of the salt extract by a 0.3 M NaCl elution of a 1.5 ml fast Q disposable column (eliminate 80-90% of nuclear proteins without topo activity); 5) fractionation of this elution fraction by anion exchange chromatography (Mono Q); topo II is eluted between 0.25 and 0.27 M NaCl and topo I between 0.26 and 0.3 M NaCl; 6) separation of topo I from topo II by superose gel filtration or glycerol gradient centrifugation.



## 2. Molecular Interactions Between DNA Intercalators and Topoisomerase II. Mechanism(s) of Enzyme Inhibition and Relationship to DNA Unwinding.

A large number of amsacrine derivatives have been synthesized. Dr. Baguley (New Zealand Cancer Research Laboratory) provided us with compounds of various DNA affinities and DNA binding sequence selectivities. In order to establish the role of intercalator-induced DNA unwinding in topoisomerase II inhibition, a new assay was designed in order to determine drug-induced DNA unwinding under topoisomerase II reaction conditions. This DNA unwinding assay uses the property of mammalian (mouse L1210) topoisomerase I to relax supercoiled DNA. Topo I induced-DNA relaxation is a direct measure of intercalator-induced DNA unwinding (Pommier et al. Nucleic Acid Res., 1987). Concentrations of 9-aminoacridines derivatives yielding similar DNA unwinding were then assayed for topo II inhibition. Neither 9-aminoacridine, nor the isomer of m-AMSA, o-AMSA inhibited L1210 topo II. Therefore DNA unwinding does not appear to be the only mechanism of enzyme inhibition. Two amsacrine derivatives with different DNA sequence selectivity of binding (G.C. vs A.T.) were also compared. Both compounds gave similar cleavage patterns and these cleavage patterns were identical to those induced by m-AMSA and the other 9-aminoacridine derivatives. However, cleavage patterns of 5-iminodaunorubicin and ellipticinium were different. All drug-induced cleavage sites corresponded to cleavage sites produced by the enzyme in the absence of drugs. These observations suggest that topo II inhibition does not result from enzyme trapping at sites of drug intercalation, but rather from drug binding within topo II-DNA complexes (Pommier et al., Biochem. Pharmacol., in press). Furthermore, the fact that ethidium bromide and the bifunctional intercalator, ditercalinium, which have high DNA binding constants and wide unwinding angles per intercalated molecules, destabilize topo II-DNA complex (Markovits et al., Cancer Res. 1986) suggests that pronounced DNA unwinding actually inhibits topo II stabilization by anti-cancer drugs.

## 3. Topoisomerase I Inhibition by DNA Intercalators.

In the course of studying the DNA unwinding potencies of amsacrine derivatives, we found that intercalators inhibited topoisomerase I-mediated DNA relaxation. This inhibition was detectable at low topo I concentrations and in kinetics experiments. Topo I inhibition was not associated with DNA single-strand breaks. DNA unwinding seemed to be a major determinant of topo I inhibition. However, m-AMSA was more potent than other 9-aminoacridine derivatives at equal DNA unwinding. Topo I inhibition could play a role in the cellular and anti-tumor effects of DNA intercalators.

## 4. DNA Sequence Localization of Drug-induced Topoisomerase II-DNA Complexes in Purified SV40 DNA.

Mapping of topoisomerase II-induced DNA breaks is an important step in understanding the mechanisms of topo II inhibition by anticancer drugs. SV40 DNA was end-labeled with [<sup>32</sup>P] at various restriction sites (Ban I, Hpa II, Taq I). This DNA was reacted with purified mouse leukemia L1210 topo II in the absence or presence of various antitumor drugs and reaction products were run into agarose and sequencing gels. Gels were dried and autoradiography performed. Topo II-mediated DNA breaks were localized by comparing the DNA



fragment sizes to that of  $\lambda$  Hind III EcoRI restriction fragments (agarose gels and computer analysis developed in the laboratory) and Maxam and Gilbert sequencing reaction products (sequencing gels). Autoradiography of agarose gels allowed the mapping of topoisomerase II-induced DNA cleavage sites (within 50 bp) within the whole SV40 genome. ATP (1 mM) enhanced enzyme-induced DNA cleavage in the absence or presence of drugs. All topo II inhibitors enhanced enzyme-induced DNA cleavage at sites that were already cut in the absence of drugs. Each class of topo II inhibitor produced a specific cleavage pattern which corresponded to a specific enhancement of certain enzyme cutting sites. In the case of intercalators, the cleavage pattern was specific of the chromophore moiety of the drug. Therefore, one can describe an acridine, an anthracycline and an ellipticine pattern of enhancement. Similar cleavage patterns were produced by m-AMSA in linear and supercoiled DNA, which suggests that torsional strain does not significantly alter topo II cleavage sites. Micrococcal nuclease also induced selective DNA cutting, whose pattern was closely similar to that induced by m-AMSA and topo II. This result suggests that mammalian topo II binds and recognize some special features of DNA structure. Autoradiography of sequencing gels showed that enzyme-induced cleavage sites were DNA sequence specific, although no obvious consensus could be derived. Analysis of DNA cleavage sites in the two 72 bp tandem repeats showed that most cutting sites were similar inside the repeats but that some differences were observed at the boundary region (within 10 bp). Therefore, topo II-induced DNA cleavage could be influenced by the DNA sequence around the cutting sites and topo II binds probably to DNA around the potential cutting sites.

##### 5. Effects of Minor Groove DNA Binding Drugs Upon Purified L1210 Topoisomerase II.

Polyamines are found in mammalian cell nuclei are millimolar concentrations. They seem to be involved in DNA replication and transcription. Because it is also the case of DNA topoisomerases, the interactions between polyamines (spermine [SPM] and spermidine [SPD]) and topo II were investigated. Both SPM and SPD induced topo II-DNA complex formation at 0.1-1 mM concentrations. These complexes had the unusual characteristics not to be associated with DNA cleavage. Under these conditions topo II-mediated DNA relaxation and catenation were stimulated. At and above 1 mM SPM, topo II-DNA complex formation and enzyme catalytic activity were inhibited. The production of m-AMSA- and VP-16-induced topo II-mediated DNA break was not changed quantitatively by SPD and only reduced at and above 1 mM SPM. Drug-induced cleavage sites were mapped within [ $^{32}$ P]-end labeled SV40 DNA. 1 mM SPM had a sequence selective effect by reducing DNA cleavage at most sites and increasing cleavage at some specific sites whose location was identical for m-AMSA and VM-26. The location of these regions is being analyzed. Our observations show that polyamines can modulate topo II activity and inhibition by anticancer drugs in a concentration-dependent manner.

Distamycin A is also a polyamine derivative which binds DNA minor groove. However, distamycin is a cytotoxic antibiotic and its DNA binding has been characterized by crystallography, NMR spectroscopy and DNA footprinting. Distamycin binds 4-6 base pairs which are either adenine or thymine. Drug-induced topo II-mediated DNA cleavage was markedly changed by distamycin A (1-10  $\mu$ M). This change consisted in both a suppression of previously enhanced



topo II cleavage sites and in a pronounced enhancement or appearance of new sites that were at identical location for m-AMSA; VM-26 and 5-iminodaunorubicin. The enhancement and suppression sites were mapped by DNA sequencing gel electrophoresis in SV40 DNA and distamycin binding determined by DNA footprinting. Suppression seems to occur at sites of distamycin binding and enhancement in 20-30 bp DNA segments wherein no distamycin binding took place. Propagated distortions of the DNA helix by bound distamycin molecules could be responsible for the enhancement effect. However, the nature of these distortions is yet unknown.

Proposed Course:

1. Complete the genomic distribution mapping of drug-induced topo II cleavage sites in SV40 DNA. Such a study could be further correlated with results obtained in SV40 infected cells (minichromosomes) treated with antitumor drugs.
2. Determine the relationship between distamycin binding to DNA and topo II modulation.
3. Study topo I inhibition by camptothecin analogs and its relationship to antitumor activity. Dr. Monroe Wall (Research Triangle Park, NC) will provide us with synthetic analogs of camptothecin modified on either cycle A or E and for which he will determine cytotoxic and antitumor activities. Camptothecin-induced topo I inhibition will be analyzed by studying the inhibition of topo I-mediated DNA relaxation and mapping DNA cleavage sites in purified SV40 DNA.

Publications:

1. Markovits, J., Pommier, Y., Mattern, M.R., Esnault, C., Roques, B.P., Le Pecq, J.B., and Kohn, K.W.: Effects of the bifunctional antitumor intercalator ditercalinium on DNA in mouse leukemia L1210 cells and topoisomerase II. Cancer Res. 46: 5821-5826, 1986.
2. Pommier, Y., Covey, J.M., Kerrigan, D., Markovits, J., and Pham, R. DNA unwinding and inhibition of mouse leukemia L1210 DNA topoisomerase I by intercalators. Nucl. Acids Res., in press.
3. Pommier, Y., Covey, J.M., Kerrigan, D., Mattes, W., Markovits, J., and Kohn, K.W.: Role of DNA intercalation in the inhibition of purified mouse leukemia (L1210) DNA topoisomerase II by 9-aminoacridines. Biochem. Pharmacol., in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06170-03

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Isolation of Human Variant Histone Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Christopher Hatch Senior Staff Fellow LMPH NCI

COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT, NCI

## SECTION

Chromosome Structure and Function

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 0.1 PROFESSIONAL: 0.8 OTHER: 0.4

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects  (b) Human tissues  (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our stated objective has been to isolate the genes for the human histone H2A family, in particular the H2A.Z gene.

The H2A.Z gene has been successfully isolated from both human cDNA and genomic DNA libraries. The homologous gene has also been isolated from bovine and rat cDNA libraries. The DNA of each of these isolates has been subcloned and (partially) sequenced. The DNA sequence of each of the H2A.Z cDNA clones has been shown to encode the H2A.Z polypeptide. Coupled transcription-translation of the H2A.Z cDNA's and analysis of the resultant polypeptide products on a two-dimensional gel electrophoresis system has demonstrated that these cDNA clones are fully representative of the coding sequence of the H2A.Z gene. Genes for the major H2A variant forms, H2A.1 and H2A.2, have been found in a human genomic library. In addition, we are actively searching for the gene of the minor H2A variant, H2A.X, in both human cDNA and genomic DNA libraries.

By studying the similarities and differences in the primary DNA structure and genomic organization of the (two) major and (two) minor (basal) histone H2A variant genes we will gain a knowledge of the underlying mechanism(s) of cell cycle dependent and independent regulation of gene expression, and further, what role(s) the basal histone variants might have in the modulation of chromosome structure and function and in the control of cell cycling and proliferation.



Project DescriptionIntroduction:

The regulation of the various histone variants differ greatly with respect to the rates of protein and DNA synthesis. Isolation of the genes for the variant histones will help elucidate these regulatory mechanisms.

Objectives:

Isolation and characterization of the human histone H2A gene family. Particular emphasis to be put into the study of the gene for H2A.Z.

Methods:

1. Recombinant DNA techniques
2. Use of synthetic oligonucleotides as probes and as primers for chain elongation in DNA sequencing reactions.
3. Rapid RNA methodology developed in this laboratory.

Major Findings and Accomplishments:

Our primary effort has been towards the isolation of the genes encoding the minor variant form of H2A, H2A.Z. The isolation of non-S-phase histone genes has been difficult. This problem is most acute in the case of the gene for histone H2A.Z., since the amino acid sequence of this polypeptide has been found to be quite divergent from that of the other histone H2A sequence while it appears that the amino acid sequence of H2A.Z is conserved in a wide variety of species, the nucleotide sequences encoding this polypeptide may be more divergent between species. An H2A.Z related gene, called H2A.F, has been isolated from chicken, but cross-hybridization of this gene to homologous sequences in human, mouse, or sea urchin DNA was not found by these investigators.

Our first efforts to isolate the human H2A.Z gene involved the use of oligonucleotides which had been designed from the codons for contiguous amino acids in the H2A.Z polypeptide to search a human genomic DNA library. In parallel the DNA of the related chicken H2A.F gene was utilized as a heterospecific probe for the human H2A.Z gene. Both of these hybridization probing methods failed to allow isolation of true H2A.Z gene clones.

We then made several important changes in approach. Firstly, the messenger RNA transcribed from the chicken H2A.F gene is thought to be polyadenylated. Similarly, a minor variant of the human H3 gene, H3.3, has also been found to be polyadenylated. So although the mRNA's from the major histone variant genes are not polyadenylated, it appears likely that most or all mRNA's transcribed from the basal histone genes are polyadenylated and should therefore be present in cDNA libraries. The sequence of interest will be enriched in relative proportion by at least several orders of magnitude in a cDNA library as compared to a genomic library. Additionally, the mRNA's of the major (S-phase) histone variants are not polyadenylated and



therefore should not be present in a typically-prepared cDNA library. The possibility that the major histone variant sequences might provide related, competing sequences for hybridization can thusly be minimized. As a further precaution, the cDNA library to be searched can be made from polyadenylated mRNA from cells known to be in a non-dividing or quiescent state and therefore transcribing only very low levels of the major (S-phase) histone variant mRNA's, but the normal, albeit low, levels of minor (basal) histone variant mRNA's, like H2A.Z and H2A.X. We searched cDNA libraries made from rat brain and bovine brain for sequences that might weakly cross-hybridize with the chicken H2A.F gene. Hybridization-positive clones were isolated from the bovine cDNA library. Positive cDNA clones containing putative H2A.Z sequences of 900 and 1300 nucleotides were isolated. Hybridization-positive clones were not found at this stage in the rat brain cDNA library. A similar search of a human lymphocyte cDNA library failed to yield any hybridization-positive clones. However, using the two newly isolated bovine H2A.Z cloned DNA's as hybridization probes, putative H2A.Z clones were isolated from both the rat brain cDNA library and the human lymphocyte cDNA library. The rat cDNA's isolated were either 1400 or 1800 nucleotides in length while the only length of cDNA for the human H2A.Z found was 1050 nucleotides. Each of the above cDNA's has been subcloned into a 'phagemid' vector (Bluescript M13 of Stratagene Cloning Systems, San Diego, California) which allows for Sanger-type sequencing of the DNA in either single or double-stranded form. These vectors also allow for transcription of the subcloned DNA. Sequencing of the bovine and human cDNA's is nearing completion and the coding regions of the observed DNA sequences are those predicted for the transcription-translation of the H2A.Z polypeptides. The polypeptides produced by coupled transcription-translation of the cDNA subclones have been found to migrate precisely to the position of H2A.Z in a two dimension gel system developed by West and Bonner for accurate identification of all histone subspecies.

The gene for H2A.Z has been isolated in cloned form from a human genomic DNA library. The DNA of these clones containing the human H2A.Z is being subcloned into the phagemid vectors for subsequent sequence analysis and comparison to the human DNA sequence. We have also isolated from a human genomic DNA library clones which contain the genes for the major H2A variants, H2A.1. and H2A.2. These genes are linked to the genes for histones H2B and H4. We are at present analyzing these clones for the presence of the minor H2A variant, X.

#### Conclusions and Proposed Course:

Knowledge of the gene structure for the minor histone variant, H2A.Z, in both its genomic and cDNA forms will provide a means to an understanding of the regulation of its expression. In so doing we will learn why, in contrast to the major (S-phase) histone variant genes, the minor (basal) histone genes are expressed constitutively at low levels throughout the cell cycle. We also hope to learn whether or not these results extend to the gene for the minor H2A variant X.

We intend to do deletion and *in vitro* mutagenesis analysis of the regulatory regions of these genes in order to delineate the critical regulatory sites. In parallel we will look for proteins which specifically bind to the regulatory



sites of these genes. The regulatory regions of the major and minor variant H2A genes will be interchanged to assay the effect of cell cycle-dependent transcription regulatory elements on cell cycle-independent coding regions and vice versa.

A collaboration will be arranged to localize the minor variant genes to particular chromosomal loci by *in situ* hybridization. This will allow us to determine whether or not there is any genomic linkage between the major and minor histone variant genes. At present only the major histone variant genes are known to be clustered and they are localized on two chromosomes, numbers 1 and 6, in human cells.

Publications:

None



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06171-03 LMPH

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Synthesis and the Control of Cell Proliferation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William Bonner Head, Chromosome Structure and Function Section LMPH NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT, NCI

SECTION

Chromosome Structure and Function

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0	0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our objective is to understand the relationship of various cell states and the transitions between them as reflected in the control of histone and chromatin biosynthesis. One phenomenon which we have studied is the differential sensitivity of the growth and chromosome cycles to the rate of protein synthesis. The chromosome cycle seems to be relatively invariant even though other results show that DNA synthesis is inhibited in parallel to the inhibition of protein synthesis. To resolve this apparent paradox, we studied histone and DNA synthesis under different conditions of protein synthesis and cell growth.

We have found and characterized a phenomenon which we have named chromosome cycle compensation. As protein synthesis is inhibited, the mRNA level of S-phase histones rises to compensate for the inefficiency of protein synthesis. There is evidence in the literature that normal and tumorigenic cells may differ significantly in their level of chromosome cycle compensation. Understanding this mechanism may lead to some insights into the growth characteristics of normal and tumorigenic cells.



Project DescriptionIntroduction:

The biochemical mechanisms which regulate cell growth remain largely unknown. For example it has been widely documented that as cell growth is slowed, the chromosome related activities and cell cycle phases are relatively unaffected. The biochemical basis for the phenomenon was unknown. Our studies this year have resulted in an explanation for this phenomenon in CHO cells.

Objective:

- 1) The development of a greater understanding of the relationship of the mechanisms regulating chromatin biosynthesis to the molecular mechanisms regulating cell proliferation.
- 2) A characterization of the responses of these regulatory mechanisms to the development of new regimens or compounds that might control inappropriate proliferation of transformed cells.

Methods:

- (1) Discontinuous electrophoretic separation of histones including direct loading of histone extracts and two dimensional electrophoresis. (Methods developed in this laboratory).
- (2) Synchronization of cell lines, particularly human Hela cells and Chinese hamster ovary cells for studies on cell cycle.
- (3) Maintenance of cells and nuclei in viable non-dividing states using modified and defined media.
- (4) Isolation and analysis of mRNA from different parts of the cell cycle or from quiescent cells, using methodology developed in concurrent project.
- (5) FACS analysis of cell cycle distributions including the BrdU antibody technique for measuring S phase cells.

Major Findings and Accomplishments:Relationship Between Protein Synthesis Inhibition, the Chromosome and Growth Cycles and the Levels of Histone mRNA and Chromatin Synthesis: An Explanation for the Relative Invariance of the Chromosome Cycle

The information reported in the literature concerning the regulatory mechanisms between DNA and protein synthesis has generally been obtained using high concentrations of inhibitors which slow either DNA or protein synthesis to 1-5% of their control level and which prevent cell growth. During the course of our work with protein synthesis inhibitors we noticed that our results on the regulation of chromatin biosynthesis around the cell cycle presented us with an intriguing paradox. That is, the rate of cell growth ( $G_1 + S + G_2 + M$ ) was apparently inhibited in proportion to the inhibition of protein synthesis but



the rate of progression of cells through the chromosome cycle ( $S + G_2 + M$ ) apparently was not. To attempt to resolve this paradox, we examined the effect of the rate of protein synthesis on the rates of synthesis of the two major components of chromatin, histone proteins and DNA, in CHO cells. We found that immediately after the inhibition of protein synthesis with low doses of cycloheximide, total protein, histone protein and DNA syntheses were inhibited proportionately. However, within a few hours the rates of synthesis of S-phase histones and DNA increased relative to the rates of synthesis of non-histone proteins and certain basal variants. Thus, proliferating CHO cells in S-phase have the ability to sense changes in the rate of protein synthesis and can compensate for any decreases in the rates of chromatin replication by increasing specifically the rates of synthesis of the two major components of chromatin, histone proteins and DNA. We call this process chromosome cycle compensation. The mechanism for this compensation involves a specific increase in the level of histone mRNAs in response to the decreased rate of protein synthesis. The amount of compensation seen in CHO cells can account quantitatively for the relative invariance in the length of the chromosome cycle ( $S + G_2 + M$ ) reported for these cells. An invariant chromosome cycle coupled with a lengthening growth cycle ( $G_1 + S + G_2 + M$ ) must result in a disproportionate lengthening of the  $G_1$  phase. Thus, these results suggest that chromosome cycle invariance may be due more to specific cellular compensation mechanisms rather than the more usual interpretation involving a rate limiting step for cell cycle progression in the  $G_1$  phase ordinary known as  $G_1$ , cell cycle arrest or  $G_1$  restriction point.

Cycloheximide slows the elongation step of translation and as such may not be a natural method for regulating protein synthesis. We are currently studying more physiological methods of limiting growth such as amino acid starvation and serum deprivation to reduce growth factors. Preliminary evidence suggests that histone mRNA levels can vary in response to changes in amino acid as well as growth factor levels in the medium.

There is also some evidence in the literature which suggests that there may be significant differences in the regulation of chromatin biosynthesis between normal and tumorigenic cells. Currently we are comparing the effects of protein synthesis inhibition on a family of 3T3 cell lines, A31, SV40 transformed A31, benzpyrene transformed and MNNG transformed 3T3 cells, to see whether any differences in the chromosome cycle compensating mechanism can be observed. Preliminary evidence suggest that there are differences in the ability of A31 and SV40 transformed A31 cells to compensate for protein synthesis inhibition. Understanding these differences may lead to the development of protocols to protect normal cells or make tumorigenic cells more vulnerable to various treatments.

#### Significance:

There are suggestions in the literature that normal and tumorigenic cells respond very differently in terms of cell cycle distribution when their growth is slowed. Our findings provide a molecular basis for this effect and thus provides experimental framework for studying this phenomenon.



Proposed Course:

1. To characterize the effect of slow growth on chromosome cycle duration in several cell lines, particularly matched pairs of normal and transformed lines, such as mouse 3T3 and BP-3T3 or human IMR-90 and VA-13.
2. To test whether chromosome cycle compensation exists in these cells or whether there are significant differences.

Publications:

1. Wu, R.S. and Bonner, W.M.: Mechanism of differential sensitivity of the chromosome and growth cycle of mammalian cells to the rate of protein synthesis. Mol. Cel. Biol. 5: 2959-2966, 1985.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06172-03 LMPh

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Base Sequence Selective DNA Alkylation Reactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kurt W. Kohn Lab Chief LMPh NCI

Others: Ann Orr Microbiologist LMPh NCI

COOPERATING UNITS (if any)

LAB/BRANCH  
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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 0.9 PROFESSIONAL: 0.5 OTHER: 0.4

CHECK APPROPRIATE BOX(ES)

(a) Human subjects  (b) Human tissues  (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project aims at the design of new alkylating agents that would be optimized for selectivity of reaction with particular locations in a DNA sequence. A premise of this work is the possibility that the antitumor effectiveness of alkylating agents depends on their reaction with certain, as yet undetermined, sites in the genome, and that their reaction at other sites would only yield deleterious effects. The approach is to search for and achieve a structural understanding of DNA sequence selective reactions by antitumor alkylating agents. Our previous work had shown that DNA sequence selective reactions do exist for nitrogen mustards and that the nature of the selectivity can be altered by altering the structure of the nitrogen mustard. In current work, the sequence selectivities have been quantitated and analysed by computer, and plausible hypotheses have been generated of the structural bases of the selectivities. It was found that the sequence selectivity pattern of most nitrogen mustards could be explained in large part as arising from the effects of neighboring base pairs on the molecular electrostatic potential in the vicinity of the reactive guanine-N7 positions. Striking deviations from this general pattern were observed for uracil mustard and for quinacrine mustards. The unique selectivities of these two mustards was explained on the basis of structural hypotheses generated with the aid of molecular modelling using computer graphics and space-filling models. It is planned to test these hypotheses by the design and synthesis of compounds that would be predicted to have enhanced or altered sequence selectivities. A later stage of this project will examine DNA sequence selectivities in chromatin and in intact cells.



Project Description:Objectives:

1. Determine the base sequence selectivities of the reactions of alkylating agents with DNA.
2. Determine the origin of the sequence selectivities in terms of molecular structure and conformation.
3. Design new alkylating agents that are optimized for selectivity of reaction with particular DNA sequence regions.

Methods:

1. High-resolution polyacrylamide gel electrophoresis of end-labeled DNA restriction fragments previously reacted with alkylating agents and chemically cleaved at sites of guanine-N7 alkylation.
2. Quantitative densitometry and computer analysis of autoradiograms of electrophoretic gels to determine corrected reaction intensities.
3. Molecular modelling by computer graphics.

Major findings:

We have previously reported that nitrogen mustards react preferentially at certain guanines in DNA and that the preferred sites may depend on the structure of the nitrogen mustard (Mattes, Hartley and Kohn, NUCL ACIDS RES 14:2971-2987, 1986). We have now carried out quantitative determinations of relative reaction rates and have formulated structural hypotheses of the origins of the reaction selectivities.

Restriction fragments of pBR322 or SV40 DNA, 32P-labeled at a 3' or 5' end, were reacted with nitrogen mustards for 1 hr at 20°C. Guanine-N7 alkylations were converted to strand breaks by heating in 1M piperidine and the resulting fragments were resolved by gel electrophoresis (Maxam-Gilbert method). Band areas were determined by computer analysis of digitized densitometer scans. Corrections were applied for proximal cuts relative to the label site and for band overlap as a function of position on the gel. Baseline corrections were taken at sites at least 2 bases removed from the nearest G.

Several mustards gave reaction intensity patterns that were similar to each other. These included HN2 (bis(2-chloroethyl)methylamine), phosphoramide mustard (PM), L-phenylalanine mustard (L-PAM), spirohydantoin mustard and chlorambucil. The reaction intensities for these mustards at low ionic strength varied from a range of 10-fold to a range of 20-fold. Dimethylsulfate gave a different pattern with an intensity range of only 5-fold. The correlation coefficients for the reactions of a given mustard relative to HN2 were between 0.81 and 0.93.

The reaction intensities of the above mustards correlated with the negative



molecular electrostatic potential (MEP) induced at the G-N7 position by the nearest neighbor base pairs, as calculated by Pullman and Pullman (QUART REV BIOPHYS 14:289, 1981). The correlation coefficients were between 0.63 and 0.90.

When 2mM Mg was included in the reaction solvent, the degree of selectivity and the dependence on MEP were reduced by 40-50% for HN2 and L-PAM; 10mM Na produced smaller reductions (25-30%). PM showed no such reductions. This can be understood from the cationic nature of the reactive aziridinium intermediates of HN2 and L-PAM, as opposed to the zwitterion in the case of PM.

We had previously reported that uracil mustard (UM) and quinacrine mustard (QM) produce distinctive reaction patterns, differing from those of the mustards discussed above. We have now characterized these differences in terms of base sequence and find that they can be accounted for on the basis of plausible structural hypotheses based on molecular modelling.

UM was found to react strongly at G's in sequences of the form, 5'-YGC-3' (Y = pyrimidine), which constitute weak sites in the case of the other mustards. When these sites were excluded from consideration, the reaction intensity pattern of UM correlated well with that of the other mustards. Moreover, the reaction intensities of UM at these sites were less affected by Mg than were other sites. Molecular modelling suggested that, as the reactive aziridinium group of UM approaches the DNA guanine-N7 position, the UM-O4 atom can interact with the amino group of the 3'-cytosine and counter its positive effect on MEP. The presence of pyrimidines on both sides of the G, according to Calladine's rules (Dickerson, JMB 166:419, 1983), would force the G to slip in the direction of its sugar. Molecular modelling by computer indicated that this would improve the geometry of the postulated interaction.

QM reacted at very low concentrations and exhibited an unusually wide range from weakest to strongest sites, in accord with an initial intercalative binding of the quinacrine group prior to alkylation. QM reacted most strongly with G's that are followed on the 3' side by a G or T, followed by a purine, and this effect was not reduced by Mg or Na. Molecular models indicated that this preference is plausible on the basis of intercalation of the quinacrine group between the 2nd and 3rd base pairs 3' to the guanine to be alkylated.

#### Significance:

These findings point to the possibility of design of DNA sequence specific alkylating agents. The potential clinical usefulness of alkylating agents that are optimized for sequence selectivity is indicated by the following consideration. We find that many alkylating agents react preferentially at runs of G's, the degree of preference being much greater than would be expected from the number of G's alone. Certain regions in the genome, including some oncogenes, and most notably the genome of Ebstein-Barr virus, have unusually high G contents. The fact that certain alkylating agents are among the best available clinical drugs, despite the great variety of targets with which



these compounds react, suggests that a great deal might be gained if compounds could be designed that would react mainly with the specific target that is most responsible for the antitumor action.

Proposed Course:

1. Design new nitrogen mustards on the basis of the structural hypotheses that we have developed. The compounds will be synthesized through collaborative arrangements and will be designed to test the structural hypotheses and to yield compounds with enhanced sequence specificities.
2. Test additional alkylating agent structures in order to search for clues to new structural features that may yield sequence selectivity.
3. Determine the effects of solvent composition, including physiologic counterions such as polyamines.
4. Extend these studies to DNA in chromatin and intact cells.

Publications:

None



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06184-02 LMPH

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

RNA Methodology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William Bonner Head, Chromosome Structure .. LMPH NCI  
and Function SectionOthers: Christopher Hatch Staff Fellow LMPH NCI  
Concepcion Muneses Chemist LMPH NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT, NCI

## SECTION

Chromosome Structure and Function

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: PROFESSIONAL: OTHER:  
0.2 0.2 0.3

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects  (b) Human tissues  (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have developed a set of procedures which simultaneously solubilize, denature, and stabilize the RNA present in whole cells or in cytoplasmic extracts for direct analysis by gel electrophoresis and hybridization of blotted gels. Multiple samples of tissue culture cells can be prepared for electrophoresis in less than an hour. The number of cells that can be processed for whole cell RNA is limited only by the sensitivity of detection of specific RNAs using hybridization probes. Cell preparations with high levels of RNase, such as human lymphocytes and HL60, can be processed reliably.

The method involves the stabilization of RNA in solubilized cells, cytoplasm or nuclei using various combinations of vanadyl ribonucleoside complex (VRC), bentonite, SDS, proteinase K, formaldehyde and heat, followed by resolution and specific detection of the RNA species on formaldehyde gels containing SDS. The transcripts of a number of different genes have been specifically detected using methodology to directly prepare and resolve the RNA from the cells of a variety of different tissue culture cell lines. We have recently extended the capability of this methodology to include the direct preparation and resolution of specific RNA's from the cells of solid tissues and tumors. At present, we are testing the ability of this technique to screen for and to assay the modulation of the level of messenger RNA transcribed from relevant genes, such as oncogenes.



Project DescriptionIntroduction:

In many studies it is desirable to measure relative mRNA concentrations in cells subjected to various treatments. This generally involves the time consuming and not always reliable purification of RNA from multiple aliquots of cells even before any analysis can begin. The purified RNAs are then commonly analyzed by electrophoretic separation, blotting of the separated RNA species onto nitrocellulose or nylon supports, and hybridization of the blotted RNA with specific labeled probe. These procedures result in information about the sizes and amounts of the mRNA species of interest. As alternatives to purifying RNA, several dot blotting procedures have been developed which measure mRNA levels in unresolved cytoplasmic or whole cell extracts<sup>3,4</sup>; the disadvantage inherent in these procedures is the difficulty in separating the desired signal (specific hybrid formation) from the noise (background, non-specific hybrid formation or filter binding).

Objective:

1. To develop procedures for the direct analysis of RNA in cytoplasmic and whole cell extracts.

Major Findings:1. The TurboBlot<sup>TM</sup> Processing System

In collaboration with American Bionetics, Inc., we tested a TurBlot<sup>TM</sup> processing system that improved the handling and processing of syntheses, Northern and dot blots. Significant savings in time and reagents were demonstrated. A paper describing this system was published.

2. Direct Analysis of RNA in Cytoplasmic and Whole Cell Extracts

We have developed a procedure in which many small samples of cells can quickly (in many cases in less than 1 hr) be prepared for direct electrophoretic analysis of intact whole cell or cytoplasmic RNA species. Attached cells are harvested by conventional or rapid techniques using RNase-free trypsin and serum or soybean trypsin inhibitor. RNA in the cytoplasm or solubilized whole cells is first protected from degradation by various combinations of vadadyl ribonucleoside complex (VRC)<sup>5</sup>, sodium dodecyl sulfate (SDS), proteinase K, heat, and formaldehyde, and then the various species resolved in formaldehyde agarose gels containing SDS. After ethidium bromide staining, the RNA is transferred to hybridization membranes by existing capillary or electrophoretic techniques. The procedures are straightforward, require only small amounts of cells and allow the simultaneous analysis of multiple samples. The yield of RNA is quantitative and reproducible from duplicate aliquots of cells. Samples can be easily normalized to cell number or to the relative amount of 18 and 28s rRNA.



Significance:

The methodology just developed has extremely wide applicability in areas of cell biology in which information about gene expression is needed.

Proposed Course:

1. We have adapted the methodology for the direct preparation and analysis of RNA from whole cells from cell culture to the cells of frozen tissue and frozen solid tumors. The possibility to directly analyze specific mRNA transcript levels, such as the mRNA's transcribed from oncogenes, will provide a clinically relevant and important means for rapid analysis of specific mRNA's in small tissue biopsy samples. We are at present using solid tumors (p388, L1210, and Lewis lung) from mice as a model system for study. Once the utility of this method is proven we will arrange to obtain human biopsy material.
2. Utilize the methodology to assess whether or not potential anti-tumor agents selectively target particular genes. Research conducted by other members of this laboratory (Matthes, Hartley, and Kohn) has demonstrated that particular alkylating agents may preferentially react with guanines in regions of the genome that are rich in contiguous guanines. In fact, certain nitrogen mustards have been shown to exhibit enhanced reactivity with such quanine-regions in the DNA of the c-H-ras oncogene. The technique for rapid preparation of RNA from tissue culture cells should facilitate the analysis of the ability of such alkylating agents to selectively inhibit the transcription of particular genes, such as certain oncogenes, like c-H-ras, which have been found to be expressed at elevated levels in the transformed cell state.

Publications:

1. Hatch, C.L., and Bonner, W.M.: Direct analysis of RNA in whole cell and cytoplasmic extracts by gel electrophoresis. Anal. Biochem. 162: 283-290, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM 06186-01 LMPH

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

DNA Repair in Specific Genomic Sequences

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Vilhelm Bohr Senior Staff Fellow LMPH NCI

Others: Diane Okumoto Microbiologist LMPH NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Pharmacology, DTP, DCT, NCI

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.5 PROFESSIONAL: 1.5 OTHER: 0.0

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our objectives are to study DNA repair in specific sequences of the genome with a regard to implications for carcinogenesis and cancer therapy.

A method was previously developed to study DNA repair in specific genomic sequences after UV damage. This has allowed us to study the DNA repair efficiency in a number of genes in various mammalian cells. DNA repair characteristics in genes have been compared to those in noncoding genomic sequences and to overall genome DNA repair measurements. The results suggest that DNA repair studies in genes are very important when correlating to other biological endpoints such as cellular resistance to carcinogens. Fine structure analysis of DNA repair in different regions of a gene locus suggest that preferential DNA repair exists within a 60-80 kb region which is likely to coincide with a higher order structure loop or domain in chromatin. Some review papers have recently been submitted. Lately, we have discovered that DNA repair efficiency is positively correlated with the level of transcription. Activation of genes enhance the rate with which they are repaired.



Project DescriptionIntroduction:

We have previous results demonstrating that essential genes in rodent cells and normal human cells are preferentially repaired after UV damage. In rodent cells, some genes are repaired much more efficiently than the bulk of the genome. This may explain the long standing paradox that the overall genome repair capacity of rodent cells is low, whereas their UV survival is as high as for proficiently repairing human cells: Rodent cells appear to repair efficiently only genomic regions of vital importance. In normal human cells, we have found that genes are repaired faster than the bulk of the genome, but eventually (after 24 hrs) all genomic regions are proficiently repaired. These demonstrations of preferential DNA repair mandates caution in interpreting correlations between overall DNA repair capacity and other biological parameters. We have correlated overall genome repair, repair in the dihydrofolate reductase (DHFR) gene, and UV resistance for three different cell lines: Chinese hamster ovary (CHO) cells, xeroderma pigmentosum group C (XPC) cells and normal human cells and demonstrated that determinations of DNA repair in specific genomic sequences may be more important than overall genome DNA repair measurements for correlations to other biological end points such as resistance to UV damage. Changes in preferential DNA repair could have profound effects on such parameters without noticeably altering overall genome repair levels since the vital regions only constitute a very small fraction of the genome. DNA repair may normally be regulated over the genome in a somewhat similar manner to that for transcription, and we propose that this regulation is deficient in the human DNA repair deficient syndrome XPC. We have also analyzed the genomic fine structure of DNA repair in and around the DHFR gene in CHO cells and find a region of preferential DNA repair of approximately 60-80 kb in length with maximal DNA repair efficiency at the 5' end of the gene and in its 5' flanking sequences. The size corresponds very well with proposed and measured lengths for loops or domains of higher order structure in chromatin, and suggests that DNA repair efficiency in genomic regions might reflect aspects of local chromatin structure and thus provide us with a probe for the detection of chromatin structural changes.

We have found considerable differences in the repair efficiency of different genes within the same cell. The constitutively transcribed proto-oncogene c-abl is much more efficiently repaired than the transcriptionally silent proto-oncogene c-mos. In recent experiments studying genes which can be modulated with regard to activity, results have further suggested that when metallothionein genes which are normally inactive become activated, considerable increases in DNA repair efficiencies can be detected. These findings suggest a positive correlation between DNA repair and the level of gene transcription.

We have studied the effect of the topoisomerase II inhibitor, novobiocin, on repair in the overall genome and in the DHFR gene. Whereas this compound inhibits overall genome repair, it had no effect on repair in the gene. This suggests that qualitative as well as quantitative differences exist between the "average" repair pathway in the cell and that responsible for the preferential repair seen in active genes. Also, novobiocin has been reported to inactivate



certain genes. If the DHFR gene is inactivated in the presence of novobiocin, this does not appear to affect the efficiency with which the gene is repaired. A constitutive efficient repair of essential genes is further supported by our findings that the DHFR gene is repaired with the same efficiency whether the cells are resting or actively growing, and our findings that another essential gene is repaired with the same efficiency whether fully active or inactivated to 5% of active transcription.

Objectives:

- 1) A number of human disorders have been termed DNA repair deficient syndromes. For none of these is the exact etiology known, nor is it known which aspect of the DNA repair mechanism is malfunctioning. For the disorder Cockayne's syndrome, it has been demonstrated that the rapid recovery of RNA synthesis after UV irradiation found in normal human cells is deficient. This suggests that this disease could represent a human mutation where preferential repair of active genes was deficient, and preliminary experiments in different laboratories confirm this. It seems possible that closer scrutiny of certain human syndromes by analysis of DNA repair in specific sequences could further our understanding of the pathomechanism of the disorders. We plan to investigate the preferential repair of genes including protooncogenes in patients with various forms of xeroderma pigmentosum, Blooms syndrome, hereditary retinoblastoma and other human syndromes where malfunctioning in DNA damage processing has been proposed.
- 2) It is of importance to study DNA repair patterns of other damaging agents than UV light. The adducts formed by a number of bulky agents are not distributed homogenously over the genome as is the case for pyrimidine dimers, but rather in a heterogenous fashion with a higher frequency in DNase I sensitive genomic regions. Recently, much interest has surrounded the discovery and cloning of the UVR a, b, c, d complex of proteins. This complex appears to cleave DNA at damage sites caused by a large number of different carcinogens. In a collaborative effort, we are presently using this complex of proteins in place of the T4 endo V enzyme in our repair assay along with slight alterations of the previously published method. Cells are treated with carcinogens, and it has been possible to detect damage and begin to measure frequency of adducts in genes and noncoding sequences in mammalian cells. The next step is to measure the repair after treatment with these agents. Preferential DNA repair of vital regions might be a general phenomenon which is seen after many kinds of damage.
- 3) Preferential binding and removal of compounds from active genes may have therapeutic implications. Our techniques to measure damage and repair in specific sequences using the afore mentioned UVR a, b, c, d complex represents a direct assessment of the frequency of sites of damage within a certain genomic region or gene. Anti-cancer drugs are in most instances known to interact directly with the DNA, and the frequency of such sites can thus be directly measured. This would allow us to screen a number of compounds in order to find those that bind most strongly to active genomic regions including specific protooncogenes. Drugs with high affinity for genes may have great potential for anti cancer therapy since inhibition of replication and inactivation of genes are likely to be main targets of the therapy. And since



the active parts of the genome only constitute a very minor fraction of the genome (< 1%) it might increase the therapeutical efficiency dramatically.

Publications:

1. Bohr, V.A., Phillips, D.H., and Hanawalt, P.C.: Heterogenous DNA damage and repair in the mammalian genome. Persp. Cancer Res., in press.
2. Bohr, V.A.: Differential DNA repair in the mammalian genome. Cancer Rev., in press.
3. Bohr, V.A.: Preferential DNA repair of active genes. Danish Med. Bull., in press.
4. Bohr, V.A., and Hanawalt, P.C.: DNA repair in genes. Pharmacol. Pharmacotherapy, in press.
5. Bohr, V.A., and Hanawalt, P.C. Enhanced repair of pyrimidine dimers in coding and non-coding sequences in CHO cells expressing a prokaryotic DNA repair gene. Carcinogenesis, in press.
6. Bohr, V.A., and Okumoto, D.: Analysis of frequency of pyrimidine dimers in specific genomic sequences. In Hanawalt, P.C., and Friedberg, E.C. (Eds.): DNA Repair: A Laboratory Manual of Research Procedures. New York, Marcel Dekker, Vol. II, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM-07119-08 LETM

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Studies on the Biochemical and Toxicology Pharmacology of Oncolytic Compounds

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. L. Litterst	Pharmacologist	LETM, NCI
OTHERS: T. C. McLemore	Senior Investigator	LETM, NCI
James Kelly	Senior Investigator	LMCB, NCI
David G. Johns	Chief	LMCB, NCI
W. Marston Linehan	Section Head	SB, NCI

## COOPERATING UNITS (if any)

LCCTP, DCE, (Dr. Poirier); OD, DCT, (Dr. Reed)

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Laboratory of Experimental Therapeutics and Metabolism

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## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither

(a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although the toxic effects of cisplatin on kidney have been appreciated for some time, the renal handling of cisplatin and the mechanism by which the renal toxicity occurs are still incompletely understood. These mechanisms could be more easily defined if the molecular sites of interaction of cisplatin were recognized. This project is designed to define how the kidney handles cisplatin under normal conditions and after various pretreatments or other experimental conditions. Inherent in this study is an attempt to localize the sites of interaction of cisplatin and its intracellular binding sites. This section reports accumulation rate and decay of Pt-DNA adducts in kidney and gonads, the tissue-specific covalent binding of Pt to renal proteins, and correlations between clinical toxicity observations and renal DNA adduct formation in animals. In addition, data are presented on the pharmacokinetics of dideoxynucleoside anti-viral drugs and the metabolic potential of human kidney toward 4-ipomeanol.



PROJECT DESCRIPTIONStudies on the mechanism of action of cisplatin:

Much work has been done to document the DNA binding of cisPt and to establish this as the probable mechanism of the antitumor effect. Similarly, much effort has been invested in documenting the various toxicities produced by cisPt, although the mechanisms of these effects have not yet been satisfactorily established. There is an obvious interest in attempting to establish whether there is any relation between DNA binding and renal toxicity. We have, therefore, investigated the relation between DNA binding as measured by an ELISA technique which is specific for the N-7 DNA adduct with cisPt, and various parameters of platinum toxicity and pharmacokinetics. In fasted rats renal adduct levels were nearly twice as great as in fed animals, although both fed and fasted rats had equivalent renal total platinum levels. This finding is consistent with the clinical finding that cisplatin is more toxic when administered in the morning after overnight fasting than when given in the evening following meals. Adduct levels in male kidney were greater than levels in female kidney. However, when cisPt was given to castrated female rats, Pt-DNA adduct levels were nearly twice as great as in normal females, even though total kidney platinum concentrations were similar. This finding is consistent with the differential response observed between older (post-menopausal) and younger (pre-menopausal) ovarian cancer patients. The implication is that there is a hormonal influence on Pt binding or transport. Castration had no effect on renal Pt-DNA adduct formation in males. A related organ-specificity also was noted, with testicles accumulating substantially greater amounts of DNA adducts than did ovaries. This is again consistent with the greater clinical response seen in testicular cancer patients than in ovarian patients. Finally, in both kidneys and gonads of males and females, DNA binding and whole tissue platinum content appear to be dose dependent, with a dramatic increase in binding at high cisplatin doses. Following bolus administration of cisplatin adduct levels in male and female kidney and in testes were greater on Day 2 than initially (4 hr after injection) and slowly declined for the next 2 weeks. Seven days after injection renal adduct levels were 23-31% of the maximum values and gonad adduct levels were 39-52% of maximum. This supports the clinical observation that weekly cisplatin administration is as efficacious but less toxic than more frequent dosing. Adduct levels in a solid 7 day old Walker 256 carcinosarcoma were only about 30% as high as those present in kidney from the same rats.

When multiple cisplatin injections are given, the second injection resulted in lower renal levels of adducts than were observed after the first injection. This again reflects clinical observations that cisplatin always appears to have greater toxicity on the first day of administration.

In summary, our work has shown that adducts between Pt and DNA form in target organs of the body, are highly stable, and have characteristics that correlate closely with clinical observations of cisplatin toxicity.

The reasons for the sensitivity of the kidney, relative to other organs, to toxic effects from cisPt are not well understood. We investigated the possible involvement of covalent binding in cisPt-induced renal toxicity. Subcellular



content of platinum (Pt) in nuclei (NUC), mitochondria (MITO), microsomes (MCR), and cytosol (CYT) from kidney, liver, and lung was determined before and after exhaustive extraction with mild acid and warm methanol, and Pt concentrations were expressed relative to tissue protein content.

At 5 min Pt concentration in kidney was 4 times greater than in liver and 2.5 times greater than in lung. For the next 24 hrs there was a 2-fold greater concentration in kidney than in liver. Renal Pt concentration was only 2.5X greater than lung 5 mins after treatment, and was 3-5X greater at later times. Pt concentration per mg protein in kidney subcellular fractions was 1.5 - 2X greater than in similar fractions from liver between 1-24 hrs, but 5 - 10X greater 5 mins after dosing. Kidney subcellular fractions contained 2 - 5X more covalently bound Pt than did liver fractions. However, a consistently greater percentage of liver Pt was covalently bound than was true for kidney Pt, suggesting that a critical site of Pt binding in kidney might be non-protein material such as lipids. Liver and kidney covalent binding reached a plateau at about 100% by 60 min and remained constant at 100% for MIT and MCR. However, kidney CYT covalent binding did not peak until 6 hrs after treatment, with 59% of Pt covalently bound to protein. Covalent binding of Pt to protein in lung subcellular fractions followed the same pattern as covalent binding in liver but values were quantitatively much less. Thus it appears that greater amounts of Pt are bound covalently to renal protein than to pulmonary or hepatic proteins and this binding may play an important role in the renal toxicity of *cis*Pt.

We also investigated the differences in renal covalent binding of Pt between a strain of guinea pig highly sensitive to the toxic effects of *cisplatin* (NIH pigmented) and a strain more resistant to *cisPt* toxicity (Hartley). There was more Pt covalently bound to protein in all 3 subcellular fractions from sensitive animals, but the differences were smaller than might be expected if covalent binding were the major determinant of toxicity.

#### Pharmacokinetics of Experimental Drugs: Dideoxy Nucleosides

A series of dideoxy nucleosides have been screened for in vitro activity against HTLV-III/LAV, the etiologic agent of AIDS. Prior to clinical trials of the most promising candidates, pharmacological studies were conducted to determine tissue and plasma/urinary pharmacokinetics of parent drug and/or relevant anabolic products (5'phosphates)

Dideoxycytosine, when administered to mice either orally or intravenously, had an oral bioavailability of 30-45% in fasted animals, with a distributional half time of near 10 min and an elimination half time of near 1 hr. The drug is rapidly anabolized and at 1 hr after treatment, 15-25% of parent drug remains in tissue. A similar study was conducted for 5'-fluorodideoxycytosine. The results showed an oral bioavailability that was greater than 60%, suggesting that the analog may be better absorbed and therefore better tolerated as an oral preparation rather than an IV formulation.



Preliminary distribution of dideoxyadenosine and its anabolites in mice have shown this drug to be rapidly and extensively anabolized with very high concentrations of 5'-phosphates, including the active triphosphate metabolite localized in the spleen, and pancreas, and significant quantities in the brain 1 hr after IV dosing.

Metabolism of Potential Anti-Cancer Drugs by Human Tissue (see also Z01-CM-07177-02 LETM)

4-ipomeanol (4-ipo) is an experimental anticancer drug soon to enter Phase I clinical trials. It is rapidly metabolized *in vivo* and the metabolite(s) bind covalently to tissue, presenting the possibility of various toxicities. The drug is known to be nephrotoxic under certain circumstances. In an attempt to further characterize the nephrotoxicity and to establish the possibility of using the drug to treat renal cancer, we obtained human tissue samples and studied 4-ipo binding to normal kidney and to kidney tumor. The covalent binding of 4-ipo to normal kidney and to kidney tumor are quantitatively and qualitatively similar. Of the 10 tumors studied thus far, all have the same 4-ipo metabolizing capacity found in normal kidney from the same patient. This lack of differential metabolic capability suggests that use of 4-ipo against renal tumors would produce equal binding in normal kidney as in tumor and have a significant potential to cause renal toxicity at doses that might be clinically efficacious.

Publications:

Litterst, C. L., Bertolero, F., and Uozumi, J.: The role of glutathione and metallothionein in the toxicity and subcellular binding of cisplatin. In McBrien, D. C. H. and Slater, T. F. (Eds.): Biochemical Mechanisms of Platinum Antitumour Drugs. England, IRL Press Limited, 1986, pp. 227-254.

Hubbard, W. C., Litterst, C. L., Liu, M. C., Bleecker, E. R., Eggleston, J. C., McLemore, T. L., and Boyd, M. R.: Profiling of prostaglandin biosynthesis in biopsy fragments of human lung carcinomas and normal human lung by capillary gas chromatography-negative ion chemical ionization mass spectrometry. Prostaglandins 32: 889-906, 1986.

Reed, E., Litterst, C. L., Thill, C. C., Yuspa, S. H., and Poirier, M. C.: cis-diamminedichloroplatinum(II)-DNA adduct formation in renal, gonadal, and tumor tissues of male and female rats. Cancer Res. 47: 718-722, 1987.

McLemore, T. L., Blacker, P. C., Gregg, M., Jessee, S. E., Alley, M. C., Abbott, B. J., Shoemaker, R. H., Litterst, C. L., Hubbard, W. C., Brennan, R. H., Fine, D. L., Eggleston, J. C., Mayo, J. G., and Boyd, M. R.: Intra-bronchial implantation. A method for the orthotopic propagation of human lung tumors in athymic nude mice. Chest 91S: 5S-8S, 1987.

McLemore, T. L., Liu, M. C., Blacker, P. C., Gregg, M., Alley, M. C., Abbott, B. J., Shoemaker, R. H., Bohlman, M. E., Litterst, C. L., Hubbard, W. C., Brennan, R. H., McMahon, J. B., Fine, D. L., Eggleston, J. C., Mayo, J. G., and Boyd, M. R.: A novel intrapulmonary model for the orthotopic propagation of human lung cancer in athymic nude mice. Cancer Res., in press.



Kelly, J. A., Litterst, C. L., Roth, J. S., Vistica, D. T., Poplack, D. G., Cooney, D. A., Nadkarni, M., Balis, F. M., Broder, S., and Johns, D. G.: The disposition and metabolism of 2',3'-dideoxycytidine, an in vitro inhibitor of HTLV-III infectivity in mice and monkeys. Drug Metab. & Disposition, in press.

Litterst, C. L., Weiss, R. B.: Clinical and experimental nephrotoxicity of cancer chemotherapeutic agents. In Bach, P.H., Lock, E. A. (Eds.): Nephrotoxicity in the Experimental and the Clinical Situation, London, CRC Press, in press.

Litterst, C.L., Poirier, M. C., Reed, E.: Factors influencing the formation and persistence of platinum DNA adducts in tissues of rats treated with cis-platin. In Hacker, M., Tritton, T. (Eds.): Organ Directed Toxicities of Anticancer Drugs, M. Nijhoff, Boston, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM-07162-04 LETM

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prostaglandins and Related Eicosanoids in Human Cancer

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W. C. Hubbard	Cancer Expert	LETM, NCI
Others:	T. L. McLemore C. P. Plopper C. L. Litterst M. R. Boyd G. N. Gray	Senior Investigator IPA Pharmacologist Associate Director Chemist	LETM, NCI LETM, NCI LETM, NCI LETM, NCI LETM, NCI

## COOPERATING UNITS (if any)

Johns Hopkins University School of Medicine (Dr. M. C. Liu)  
 Program Resources, Inc. (Dr. M. C. Alley)

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## PROFESSIONAL:

1.0

## OTHER:

1.0

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(a) Human subjects       (b) Human tissues       (c) Neither

(a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The profiles of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostaglandin F<sub>2a</sub> (PGF<sub>2a</sub>), prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), thromboxane B<sub>2</sub> (TxB<sub>2</sub>) and 6-keto-prostaglandin F<sub>1</sub> (6KPGF<sub>1</sub>) in lung tissue and lung carcinoma tissue were compared in relation to the histologic classification of the lung neoplasm. Eight histologic classifications of tumor tissue were employed: squamous cell carcinoma (N=18); adenocarcinoma (n=6), small cell carcinoma (n=4), mixed cell carcinoma (n=2) bronchioloalveolar carcinoma (n=2) and metastatic tumors (n=3). PGE<sub>2</sub> production was greater in all but two histologic classifications of lung tumors than in lung tissue. The biosynthesis of PGE<sub>2</sub> was less in large cell carcinoma tissue. Metastatic tumor tissue synthesized PGE<sub>2</sub> in equal quantities in comparison with lung tissue. Similar levels of PGF<sub>2a</sub> production were observed in lung and tumor tissues classified as follows: small cell carcinoma, large cell carcinoma and metastatic tumors. Higher levels of PGF<sub>2a</sub> synthesis were evident in squamous cell carcinoma, adenocarcinoma, mixed cell, bronchioloalveolar and bronchial carcinoid tissues. The most profound differences in PGD<sub>2</sub> biosynthesis were evident in comparisons of lung tissue and large cell carcinoma tissue with PGD<sub>2</sub> being higher in this tumor tissue. The profile of bisenoic prostanoids synthesized from endogenous arachidonic acid in 13 established cell lines derived from human lung carcinomas have been determined. PGE<sub>2</sub> and PGF<sub>2a</sub> were produced in all cell lines with demonstrable prostanoid biosynthesis (DMS-273, NCI-H460, NCI-H522, NCI-H358, Calu-3, Calu-6, A549). TxB<sub>2</sub> production was evident only in these cell lines originating from lung adenocarcinomas (Calu-3, A549, Calu-6). 6KPGF<sub>1a</sub> and 9<sub>a</sub>,11<sub>b</sub>-PGF<sub>2a</sub> were products in Calu-3 cells only. Our findings suggest that certain lung carcinomas selectively synthesize prostaglandins.



PROJECT DESCRIPTIONOBJECTIVES

The lung is a major organ source of prostaglandin and thromboxane biosynthesis. In addition, the lung is rich in enzymes that catalyze the rapid transformation of biologically active prostanoids either to compounds devoid of significant activity or to one or more species having biological activity different from that of the parent prostanoid. Moreover, it has been shown that PGE<sub>2</sub> production is elevated in patients with squamous carcinoma of the lung and that PGE<sub>2</sub> biosynthesis is greater in lung carcinoma tissue than in lung tissue. The profiles of prostanoid biosynthesis in normal lung tissue, in lung carcinoma tissue and in established cell lines derived from human lung carcinomas have been evaluated. Our objectives in these studies were: 1) the determination of selectivity of prostanoid biosynthesis in human lung carcinomas and 2) the quantitative comparisons of the capabilities of lung tissue and lung carcinoma tissue to synthesize prostaglandins.

I. Prostanoid Biosynthesis from Endogenous Arachidonic Acid in Lung Tissue and Lung Carcinoma Tissue from Cancer Patients

The profile of biosynthesis of PGE<sub>2 $\alpha$</sub> , PGD<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , TXB<sub>2</sub> and 6KPGF<sub>1 $\alpha$</sub>  from endogenous arachidonic acid in biopsy fragments of lung tissue and lung carcinoma tissue from patients at the time of diagnostic thoracotomy have been determined (see Z01 CM-07177-02). The patient population was subdivided into those that currently smoke cigarettes and former smokers who have abstained from cigarette smoking for >6 months. The consumption of cigarettes of smokers (n=24) was 58 + 30 (SD) packs/year. Previous cigarette consumption in current nonsmokers (n=15) was 45 + 33 (SD) packs/year.

The relative quantitative profile of prostaglandin and thromboxane biosynthesis in both patient populations was as follows: 6KPGF<sub>1 $\alpha$</sub>  > TXB<sub>2</sub> > PGD<sub>2</sub> > PGE<sub>2</sub> = PGF<sub>2 $\alpha$</sub> . The differences in the biosynthesis of individual prostanoids in lung tissue from smokers and nonsmokers were not significant.

The relative quantitative profile of prostaglandin and thromboxane biosynthesis in nonsmokers was: 6KPGF<sub>1 $\alpha$</sub>  > PGF<sub>2 $\alpha$</sub>  > TXB<sub>2</sub> > PGE<sub>2</sub> > PGD<sub>2</sub>. In smokers this profile was: TXB<sub>2</sub> > 6KPGF<sub>1 $\alpha$</sub>  = PGF<sub>2 $\alpha$</sub>  = PGE<sub>2</sub> > PGD<sub>2</sub>. Prostanoid biosynthesis was significantly higher in tumor tissue from smokers and nonsmokers were as follows: PGF<sub>2 $\alpha$</sub> ; PGE<sub>2</sub>; TXB<sub>2</sub>; and 6KPGF<sub>1 $\alpha$</sub> .

In addition to comparisons of prostanoid biosynthesis from endogenous precursor in lung tissue and lung carcinoma tissue, the profile of prostaglandin and thromboxane biosynthesis in relations to the histologic classification of the neoplasm was compared. PGE<sub>2</sub> biosynthesis was higher in tumor tissue of all histologic types of primary lung tumors than in lung tissue with one exception. PGE<sub>2</sub> production was greater in lung tissue than in large cell undifferentiated carcinoma tissue. PGF<sub>2</sub> was synthesized in greater quantities in squamous cell carcinoma, adenocarcinoma, bronchioloalveolar and mixed cell carcinoma tissues than in lung tissue. Equal quantities of PGF<sub>2</sub> were synthesized in small cell carcinoma and in large cell carcinoma tissues in comparison to lung tissue. Significant differences in PGD<sub>2</sub> biosynthesis was seen when products from lung tissue and large cell carcinoma tissue was compared. Lung tissue synthesized



greater quantities of PGD<sub>2</sub> than was produced in large cell carcinoma tissue. Correlative relationship between the histologic classification of lung carcinomas and the biosynthesis of TxB<sub>2</sub> and 6KPGF<sub>1</sub> could not be established.

In addition to determinations of individual prostanoids synthesized in human lung and lung carcinoma tissues incubates of the tissue were evaluated for the presence of 15-keto-13,14-dihydro-metabolites of PGF<sub>1</sub>, PGF<sub>2</sub> and 6KPGF<sub>1</sub> metabolites. None of these metabolites were present in detectable levels.

#### METHODS DEVELOPMENT AND EMPLOYMENT

Comparisons of prostanoid production in lung tissue and lung carcinoma tissues indicate that these tissues have different capabilities for prostaglandin and thromboxane biosynthesis. Moreover, previous studies in this laboratory (see previous annual reports) suggest that prostanoid biosynthesis may be a characteristic of certain lung tumors. Established cell lines derived from human lung carcinomas provide us with simplified experimental systems for studies of prostaglandin biosynthesis in tumor cells free of nontumor cells. The availability of an octadeuterated (<sup>2</sup>H<sub>8</sub>) analog of arachidonic acid dictated the use of an experimental design in which the stable isotope analog was employed as an exogenous substrate for the prostaglandin H synthase system in cultured cells derived from human lung carcinomas. In addition to providing definitive information of the fatty acid precursor of prostanoids biosynthesized in the cultured cells, the use of <sup>2</sup>H<sub>8</sub>-arachidonic acid as an exogenous substrate facilitated product identification. Existing analytical methods employing capillary gas chromatography-mass spectrometry were adapted for the detection of multi-deuterated 20-carbon fatty acid cyclooxygenase metabolites of arachidonic acid synthesized from <sup>2</sup>H<sub>8</sub>-arachidonic acid.

#### MAJOR FINDINGS

The profile of 20-carbon fatty acid cyclooxygenase products synthesized in 13 established cell lines have been completed. Significant prostanoid biosynthesis was observed in 8 of the established cell lines. The 8 cell lines with demonstrable prostaglandin biosynthesis were derived from large cell carcinomas (2/2 cell line; NCI-H460; A427), bronchioloalveolar carcinoma (2/2 cell lines; NCI-H322, NCI-H358), small cell carcinomas (1/4; DMS273) and adenocarcinomas (3/4; Calu-3; Calu-6; A549). Evidence for thromboxane (Calu-3, Calu-6, A549) prostacyclin (Calu-3) and PGD<sub>2</sub> production (Calu-3) was observed only in cell lines originating from lung adenocarcinomas.

#### PROPOSED COURSE

The future course of these studies will include the following priorities. First, the profile of bisenoic prostaglandin biosynthesis in additional cell lines (10 additional cell lines readily available) derived from human lung carcinomas will be explored. Second, the profile of prostanoid production in cell lines originating from other human tumors (ovarian carcinomas, renal cell carcinomas, etc) will be determined. Third, quantitation of the prostaglandin



synthase content of cell lines derived from human tumors will be determined immunohistochemically (with Dr. C.G. Plopper). Fourth, the kinetics of prostaglandin production and possible metabolism to less active species will be undertaken.

#### PUBLICATIONS

Hubbard, W. C., Litterst, C. L., Liu, M. C., Bleecker, E. R., Eggleston, J. C., McLemore, T.L., and Boyd, M.R.: Profiling of prostaglandin biosynthesis in biopsy fragments of human lung carcinoma and human lung tissue by capillary gas chromatography-negative ion chemical ionization mass spectrometry. Prostaglandins 32: 889-906, 1986.

Hubbard, W. C., Litterst, C. L., Liu, M. C., Bleecker, E. R., Mimnaugh, E. G., Eggleston, J. C., McLemore, T. L., and Boyd, M. R.: Detection and quantitation of eicosanoids by combined gas chromatography-mass spectrometry. In Walden, T. L. and Hughes, H. N. (Eds.): Prostaglandins and Lipid Metabolism in Radiation Injury, New York, Plenum Press, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM-07177-02 LETM

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Clinical Lung Cancer Research Project

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: T. L. McLemore	Senior Investigator	LETM, NCI
Others: M. R. Boyd	Associate Director	DTP, DCT
C. L. Litterst	Pharmacologist	LETM, NCI
C. Plopper	IPA	LETM, NCI
B. Coudert	Visiting Fellow	LETM, NCI
S. Adelberg	Biologist	LETM, NCI

## COOPERATING UNITS (if any)

BTB (Dr. Mayo, Ms. Abbott, Dr. Shoemaker); Johns Hopkins University School of Medicine (Drs. Eggleston, Liu); Program Resources, Inc. (Drs. Alley, Fine)

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## OTHER:

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(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The development of therapeutic modalities for the treatment of human lung cancer has, to date, been disappointing. Classification of lung tumors with regard to their metabolic potential is particularly important for developing a systematic approach for the identification of active anti-lung tumor agents. We have, therefore, initiated this project in order to develop a relevant and feasible plan for classification of human lung tumors, as well as for identification of effective new lung cancer treatment modalities. This project will interface the clinical aspects of human lung cancer with basic science and applied science technologies which are currently available in an effort to provide a comprehensive program for characterization of lung tumors based on their morphological, ultrastructural, biochemical, metabolic, and molecular genetic characteristics as well as establish in vitro and in vivo techniques for investigating potential antitumor drugs against primary human lung tumors. The initial results from our laboratories document the feasibility and relevance of this project by demonstrating: (1) considerable variation in prostanoid and 4-ipomeanol metabolism as well as cytokeratin expression among individual fresh human lung tumor specimens as well as lung tumor cell lines, (2) the ability to establish and propagate primary lung tumor cell cultures which will provide material for in vitro drug screening, establishing long term tumor cell lines, and for comparative biochemical studies, (3) the ability to propagate human lung tumors in the lungs of nude mice by novel intrabronchial and intrathoracic implantation methods which allow for improved in vivo propagation, biochemical and drug screening studies. An ultimate goal of this project is to provide a paradigm for individualized lung cancer characterization and pharmacoselective drug treatment based on the biochemical profiles of individual lung tumors and corresponding normal lung tissue.



BRIEF DESCRIPTION OF PROJECT

Primary lung cancer has become the major cause of cancer-related adult deaths in the U.S. Despite advances in treatment of other human cancers, the 5-year survival for lung cancer has remained unchanged (5% to 8%) over the past 20 years. The inadequacies of current approaches for the diagnosis and treatment of lung cancer have led us to an extensive re-evaluation of this disease entity. Recent evidence from the LETM laboratories would suggest that certain ultrastructural and biochemical features might be employed to characterize individual long term lung tumor cell lines. These preliminary data need further investigation, especially in fresh human lung tumor specimens. The current lung cancer research proposal interfaces the clinical aspects of human lung with basic science and applied science technologies in an effort to provide a comprehensive program for characterization of lung tumors based on their morphological, ultrastructural, biochemical, molecular genetic and metabolic capabilities as well as to establish in vitro and in vivo techniques for investigating potential antitumor drugs against primary human lung tumors.

OBJECTIVES

- I. The establishment of collaborative arrangements with Johns Hopkins University School of Medicine for the purpose of studying a large population of primary lung cancer patients and obtaining well characterized lung tumor specimens for study.
- II. The biochemical, metabolic, and molecular genetic characterization of human lung tumors and normal lung tissue. These presently include:
  1. Prostanoid biosynthesis and metabolism.
  2. 4-Ipomeanol (IPO) metabolism.
  3. Cytochrome P-450 isozymes: metabolism and gene expression.
  4. Conjugating and detoxifying enzymes; metabolism and gene expression.
  5. Cytokeratin expression.
- III. The establishment of a system for characterization of human lung tumors based on comparison of their morphological, ultrastructural, biochemical, metabolic, and molecular genetic properties.
- IV. Antitumor drug studies employing primary lung tumor cultures in collaboration with DTP In Vitro/In Vivo Drug Screening Project. This would include:
  1. The comparison between the responses of fresh primary lung tumor cell lines to standard antitumor agents.
  2. The comparison between the in vitro response of individual primary lung tumor cultures and the clinical response of the same patient's tumor to antitumor agents.
  3. The establishment of long term human lung tumor cell lines for use in the In Vitro Drug Screening Project.
- V. The development of in vivo models in athymic nude mice for human lung tumor propagation as well as detailed biochemical and drug screening studies.



1. Intrabronchial (i.b.) model development.
2. Percutaneous intrathoracic (i.t.) model development.
3. Development of radiographic techniques for noninvasive monitoring of lung tumors in these models.
4. Use of i.b. and i.t. models for in vivo drug screening.

#### MAJOR FINDINGS AND RELEVANCE

##### I. Biochemical and Metabolic Characterization of Human Lung Tumors and Normal Lung Tissue

###### Major Findings

###### a. Prostanoid biosynthesis and metabolism in:

1. Fresh human lung tumor and normal lung tissue specimens (see Z01 CM-07162-04 LETM for details)

2. Continuous human lung tumor cell lines (see Z01 CM-07162-04 LETM for details)

###### b. Fresh human lung tumor and normal lung tissue specimens:

###### 1. Fresh human lung tumor and normal lung tissue specimens

IPO is a pulmonary toxin currently under development as a potential antitumor agent. The rodent pulmonary cytotoxicity of IPO is mediated through its intracellular bioactivation to a highly reactive metabolite. The capacity of human lung cells to bioactivate IPO has not, heretofore, been investigated. The present studies compared IPO metabolism via quantitation of covalent <sup>14</sup>C IPO binding, in fresh lung tumor explants as well as normal lung cancer patients. A linear relationship was demonstrated for IPO covalent binding in tumor and normal lung tissue over a 45 min incubation period as well as between substrate concentration and IPO covalent binding for 0-10 mM IPO concentrations (N=10). Inhibition of covalent binding was observed uniformly for lung tumor and normal lung by 1 mM piperonyl butoxide, boiling, or placing on ice at 4°C. Mean covalent binding of IPO metabolites in normal lung and lung tumors were similar for the patients studied, with values of 374  $\pm$  37 and 394  $\pm$  43, respectively (X  $\pm$  S.E. pmoles <sup>14</sup>C IPO bound/mg protein/30 min.; p > .30). A 40-fold interindividual variation in IPO metabolism was also noted for both normal and lung tumor tissue. When IPO metabolism was compared on the basis of histological tumor cell type mixed tumors (N=2) > small cell carcinoma (N=4) > adenocarcinoma (N = 8) > squamous cell carcinoma (N= 17) > large cell undifferentiated carcinoma (N=4) > bronchioloalveolar cell carcinoma (N=2) > metastatic tumors (N=3) > bronchial carcinoid (N=1). When IPO covalent binding was compared for normal lung and tumor tissue from individual lung cancer patients, no linear relationship was observed; however, three distinct categories of patients were noted: (1) those with higher IPO metabolism in lung tumor than in normal lung, (2) those with higher IPO metabolism in normal lung than in lung tumor, and (3) patients with similar IPO CB capabilities in both normal lung and lung tumor tissues. These results suggest dysregulation of IPO metabolism in lung tissue compared with normal tissue from the same patient. Further studies will be required, however, to delineate the mechanisms related to this phenomenon, and to ascertain any possible therapeutic implications.



## 2. Continuous human lung tumor cell lines

Considerable variation was observed for  $^{14}\text{C}$  IPO bonding in 20 different human lung tumor cell lines representing a number of different histologic cell types. Highest activity was noted for the large cell-small cell mixed tumor cell type with 461 pmoles  $^{14}\text{C}$  IPO bound/mg protein/30 min (N=1). IPO binding for other histologic cell types was as follows: Bronchioloalveolar cell carcinomas, 375 + 76 (x + S.D.; N=2) classic small cell carcinomas, 335 + 357 (N=3); adenocarcinomas of the lung, 294 + 439 (N=6); adeno-squamous cell carcinomas, 264 (N=1); large cell carcinomas of the lung, 234 + 364 (N=3); muco-epidermoid carcinomas, 31 (N=1); variant small cell carcinomas of the lung, 27 (N=1); and squamous cell carcinomas of the lung, 0 + 0 (N=2). Values for  $^{14}\text{C}$  IPO binding for the different human lung tumor culture cell lines ranged from 0-1260. In addition, similar  $^{14}\text{C}$  IPO binding was noted in the original HOP 27 lung tumor specimen (309) and in the cultured tumor cell line derived from the original tumor specimen (461). These results demonstrate similar variation in IPO binding to that observed in fresh human lung tumor specimens (see above) derived from different histologic cell types.

## 3. Fresh human renal tumors and normal renal tissues. (See Z01 CM-07119-07 LETM for details)

### c. Cytokeratin expression in fresh lung tumor and normal lung tissue specimens

A library of 17 monoclonal antibodies to human keratins have been applied to tissue specimens from human lung tumors, normal human lung and fetal and adult nonhuman primate lung to determine keratin expression using immunocytochemistry. This library recognizes keratins in the full range of sizes established by Moil. Preliminary studies to establish fixation and incubation conditions for each antibody indicated that ethanol/acetic acid (72/25) fixation followed by parafin embedding was best for most antibodies. Fresh frozen specimens provided stronger antibody binding, but the poor resolution of the specimens made interpretation difficult. Optimum reaction on ethanol/acetic acid, or even paraformaldehyde-fixed specimens was produced by preceding the antibody reaction by a mild trypsin digestion. To date seven human lung tumor, five normal human lung and about 40 primate lung specimens are being evaluated. Further efforts are being made to secure additional normal human lung tissue. Preliminary results suggest that some of the keratins are not expressed at all in primate lung including keratins 1,3,5,6,8,10 and 18). Some are restricted to specific cell types, such as 13 in basal cells and 17 in ciliated cells and 18 in type 2 cells. Generally, most of the keratins expressed in normal lung are also present in lung tumors. However, some tumors may express keratins not normally seen in adult lung, but further studies will be required because of the difficulty of obtaining a large number of high quality normal human lung tissues. Additional evaluation of a group of six new tumors and six normal lung specimens should help clarify the situation. Confirmation of keratin expression will be carried out using gel electrophoresis and immunoblotting of keratins from lung tissue. It is anticipated that typing of individual lung tumors with respect to cytokeratin expression will be feasible during studies performed in the next year.



## II. In Vitro Propagation of Fresh Human Lung Tumors

To date, 73 human lung tumor primary cell cultures have been established representing 18 adenocarcinomas, 30 squamous cell carcinomas, 6 small cell carcinomas, 8 large cell undifferentiated carcinomas, 3 mixed cell tumors, 2 bronchiolo-alveolar cell carcinomas, 2 carcinoid tumors, 1 undifferentiated carcinoma and 3 metastatic tumors. Six new continuous long term human lung tumor cell lines, designated as Hop 7, Hop 14, Hop 18, Hop 19, Hop 22 and Hop 27, have been successfully established from the first 30 primary cultures. This represents a 20% overall success rate for establishing tumor cell lines. These cell lines include 2 squamous cell carcinomas (Hop 7 and 14), 2 large cell undifferentiated carcinomas (Hop 18 and 22), 1 adenocarcinoma (Hop 19) and 1 small cell-large cell carcinoma (Hop 27). Histologic, biochemical and ultrastructural investigations are currently ongoing to compare the lung tumor cell lines with the original tumor specimens from which they were derived.

## III. New Approaches to the In Vivo Propagation of Human Lung Tumors

### a. i.b. Model

A novel model for the propagation of human lung tumor cells in the bronchiolo-alveolar regions of the right lungs of athymic NCr-nu/nu mice via an i.b. implantation procedure has been developed. Over 1000 i.b. implantations have been performed to date, each requiring 3-5 min. for completion and having a surgery-related mortality of approximately 5%.

#### 1. Propagation of human lung tumor continuous cell lines

The i.b. model has been successfully employed for the orthotopic propagation of 4 established human lung cancer cell lines including: an adenosquamous cell carcinoma (NCI-H125), an adenocarcinoma (A549), a large cell undifferentiated carcinoma (NCI-H460) and a bronchioloalveolar cell carcinoma (NCI-H358). When each of the 4 cell lines were implanted i.b. using a  $1.0 \times 10^6$  tumor cell inoculum,  $100 + 0\%$  ( $x \pm S.D.$ ) tumor-related mortality was observed within 9 to 61 days. In contrast, when the conventional subcutaneous (s.c.) method for implantation was employed, at the same tumor cell inoculum, only minimal ( $2.5 + 5\%$ ) tumor-related mortality was observed within 140 days ( $p < 0.001$ ). Similarly, when a  $1.0 \times 10^5$  or  $1.0 \times 10^4$  cell inoculum was employed, a dose-dependent, tumor related mortality was observed when cells were implanted i.b. ( $56 + 24\%$  or  $25 + 17\%$ ) as compared with the s.c. method ( $5 + 5.7\%$  or  $0.0 + 0\%$ ) ( $p < 0.02$  and  $p < 0.05$ , respectively). Most ( $>90\%$ ) of the lung tumors propagated by i.b. implantation were localized to the right lung fields as documented by necropsy and/or high-resolution chest roentgenography techniques which were developed for these studies.

#### 2. Propagation of fresh human lung tumor cell suspensions

The intrapulmonary model was also employed for establishment and propagation of xenografts derived directly from enzymatically digested, fresh human lung tumor specimens obtained at the time of diagnostic thoracotomy. Approximately 35% (10/29) of the fresh primary human lung tumor specimens and 66% (2/3) of tumors



metastatic to the lung were successfully propagated i.b. at  $1.0 \times 10^6$  tumor cell inoculum; whereas, only 20% (1/5) of the specimens were successfully grown in vivo via the s.c. route from a  $1.0 \times 10^7$  tumor cell inoculum. Primary human lung tumors successfully propagated i.b. included: 3 squamous cell carcinomas, 3 adenocarcinomas, 1 small cell carcinoma, 1 large cell carcinoma, 1 large cell-small cell carcinoma and 1 bronchioloalveolar cell carcinoma. Our experience with this in vivo intrapulmonary model for the orthotopic propagation of human lung tumor cells is consistent with the view that organ specific in vivo implantation of human tumors facilitates optimal tumor growth. This new in vivo lung cancer model may substantially facilitate future studies of the biology and therapeutics of this catastrophic disease.

b. i.t. Model

A new model for the orthotopic propagation of human lung tumor continuous cell lines in the pleural space of athymic nude mice has been developed employing a i.t. implantation technique. Over 300 i.t. implants have been performed in our laboratory to date, each requiring approximately 1 minute for completion and demonstrating a surgery-related mortality of approximately 8%.

1. Propagation of human lung tumor continuous cell lines

This model has been successfully employed for the propagation of 5 different human lung tumor cell lines representing adenocarcinoma, small cell carcinoma, large cell undifferentiated carcinoma, adenosquamous cell carcinoma and bronchioloalveolar cell carcinoma histologic cell types. 100% tumor-related mortality was observed in those animals implanted i.t. with the 5 different cell lines at a  $1.0 \times 10^6$  cell inoculum and subsequently followed for a maximum of 180 days (N=51). In contrast, only minimal (5%) tumor-related mortality was observed when athymic nude mice were implanted subcutaneously (s.c.) with similar tumor cell inoculum and followed for the same time period. (N=50; p<0.001 nonpaired, one-tailed student t-test). In addition, a dose dependent effect was noted for survival in animals implanted i.t. at  $1.0 \times 10^5$  (N=52) or  $1.0 \times 10^4$  (N=51) tumor cell inocula. Histologic appearance of the 5 lung tumor cell lines implanted i.t. or s.c. were similar and were representative of the current lung tumor cell lines from which they were derived. Approximately 30% of the tumors implanted i.t. grew in the chest wall and/or lung parenchyma as well as in the pleural space of nude mice.

2. Propagation of fresh human lung tumor specimens

Forty-three percent (3/7) of the fresh human primary lung cancers or low passage xenografts obtained from primary human lung tumors were successfully propagated i.t. These included 2 squamous cell carcinomas and 1 large cell-small cell mixed tumor. Histologic appearances of these tumors were similar to the original human lung tumor specimens from which they were derived.

c. Comparison of i.b., i.t. and s.c. Models

The propagation efficiencies, growth patterns, and histologic appearances of 6 continuous lung tumor cell lines implanted i.t. and i.b. were compared with the conventional s.c. implantation method at three different tumor cell inocula



(N=184 i.b., N=185 i.t. and N=180 s.c.). A tumor-related mortality of 100% was noted when the 6 different lung tumor cell lines, representing adenocarcinoma, adenosquamous carcinoma, large cell undifferentiated carcinoma, small cell carcinoma, and bronchioloalveolar cell carcinoma, were implanted i.b. at a  $1.0 \times 10^6$  tumor cell inoculum. A similar tumor-related mortality (92%) was observed when a  $1.0 \times 10^6$  tumor cell inoculum was implanted i.t., ( $p > 0.10$ , nonpaired one-tailed student's t test) whereas minimal (5%) tumor-related mortality was noted when cells from the 6 different cell lines were implanted s.c. at a similar inoculum ( $p < 0.001$  in all instances). In addition, a dose dependent, cancer-related mortality was noted for either the i.t. or i.b. implantation when lower ( $1.0 \times 10^5$  or  $1.0 \times 10^4$ ) tumor cell inocula were employed. Comparison of histologic characteristics and growth patterns of tumors propagated employing the 3 implantation techniques revealed similar for all 3 propagation methods and in all instances, histologic appearances of the tumors studied were representative of the current tumor cell lines from which they were derived. Tumor-specific selectivity was also noted for the i.t. model in that only 50% cancer-related mortality was observed for one bronchioloalveolar cell carcinoma cell line, NCIH358, while all 5 of the other cell lines tested (including another bronchioloalveolar cell carcinoma cell line NCI-322) demonstrated 100% tumor-related mortality when implanted i.t.. In contrast, NCI-H358 demonstrated 100% mortality when implanted by the i.b. method. When 5 nonpulmonary human tumor cell lines were propagated i.b. or i.t., there was considerable variability in tumor-related mortality depending on the tumor type. The U251 glioblastoma, the LOX amelanotic melanoma and the HT-29 colon adenocarcinoma cell lines demonstrated  $>70\%$  tumor related mortality when implanted i.t. or i.b. at a  $1.0 \times 10^6$  tumor cell inoculum (N=10 for each cell line implanted i.b. or i.t.). In contrast, minimal tumor-related mortality was noted for the MCF-7 breast adenocarcinoma adriaycin resistant or the Ovcar 3 ovarian carcinoma cell lines when these were implanted i.b. or i.t. at a  $1.0 \times 10^6$  tumor cell inoculum (N=10 for each cell line implanted i.b. or i.t.). These data further support the concept of organ site specificity. Approximately 30% of the lung tumors propagated i.t. grew in the chest wall and/or in the lung parenchyma in addition to growing in the pleural space. In contrast, tumors propagated i.b. grew predominantly in the lung parenchyma. These data support the concept that the intrapulmonary model has selective advantages over the i.t. model for the study of pulmonary cancers. However, both orthotopic models appear to be superior to the conventional s.c. model for the propagation as well as the study of human pulmonary carcinomas *in vivo* and should be advantageous for future studies of lung cancer biology and developmental therapeutics.

d. Development of x-ray technology for evaluation of i.b. and i.t. propagated tumors

A low dose, high resolution mammography radiographic device has been employed to successfully develop roentgenographic techniques for noninvasive evaluation of i.b. or i.t. propagated tumors in athymic nude mice. A library of normal athymic nude mouse x-rays have been established and the radiographic technique have been successfully employed to identify small (1 mm) tumor nodules in the lungs of nude mice. Sequential tumor progression can also be monitored utilizing these techniques. Studies are currently underway to follow regression or progression of tumors following i.b. or i.t. implantation and subsequent anti-tumor chemotherapy treatment after radiographic identification of a tumor mass (see below).



e. Use of the i.b. model for antilung tumor drug screening

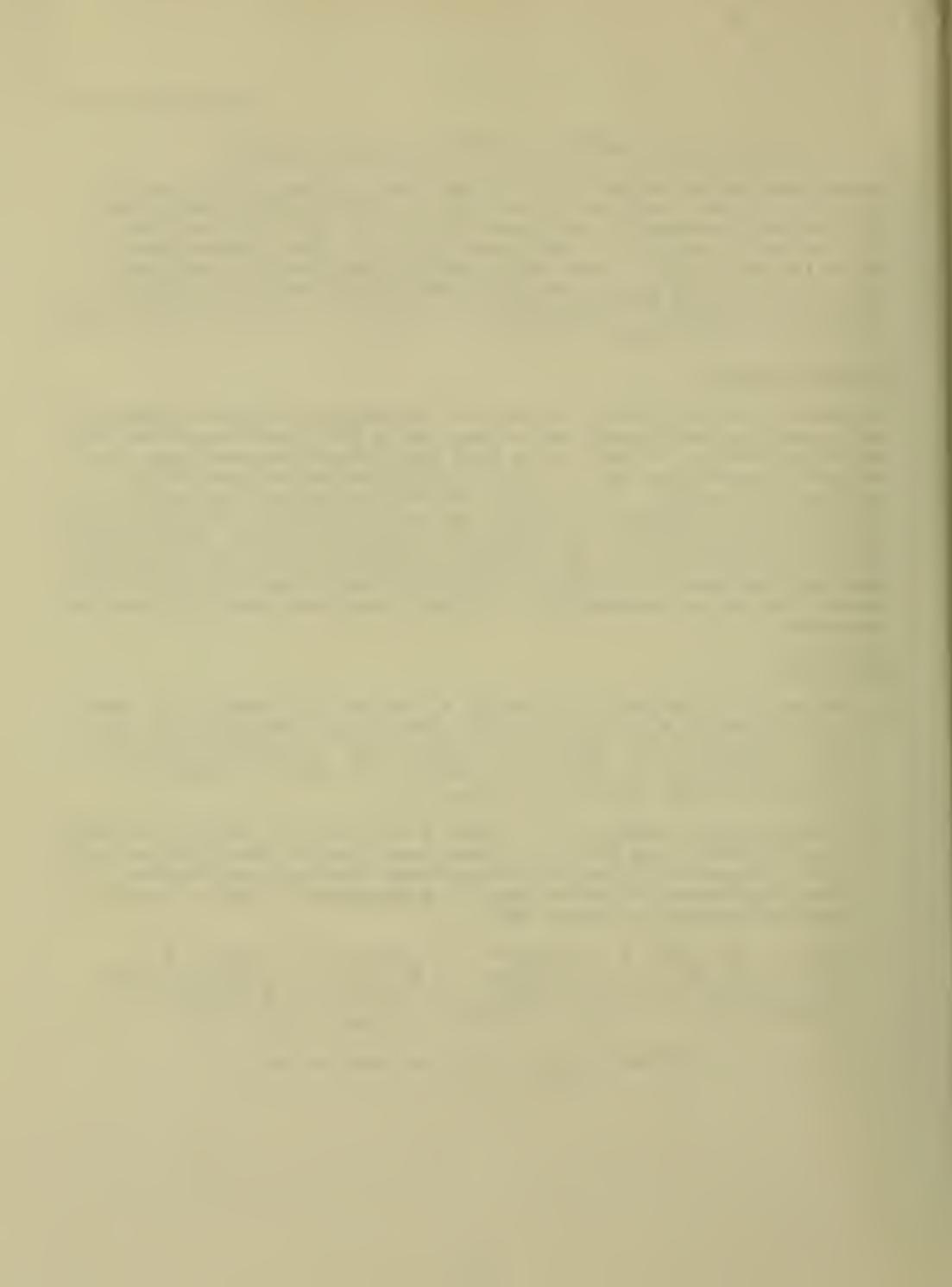
Preliminary studies employing the i.b. model have demonstrated 20% long term survival in animals implanted i.b. with the NCI-H358 bronchioloalveolar cell carcinoma and subsequently treated with 30 mg/kg IPO after tolerance induction with low dose (1 mg/kg) IPO. This tumor has been previously demonstrated to metabolize IPO in vitro. These initial results demonstrate the usefulness of the i.b. model for in vivo screening and combined with noninvasive x-ray procedures could represent an animal model for lung cancer similar to that observed clinically in a patient population.

FUTURE DIRECTIONS

The studies performed to date indicate that the original objectives proposed for this project are both feasible and relevant and we will therefore continue the project as outlined. In addition, a small group of other relevant human tumors and normal human tissues will be investigated by our current protocols for comparative evaluations. By exploring the relationships among the morphological, ultrastructural, biochemical, metabolic and molecular genetic characteristics of individual lung tumors with the in vitro and in vivo response of these tumors to specific antitumor agents, a paradigm for individualized antitumor chemotherapy might be established. This might eventually provide an approach to lung cancer characterization and treatment that is an improvement over existing alternative approaches.

PUBLICATIONS

1. McLemore, T.L., Blacker, P.C., Gregg, M., Jessee, S.E., Alley, M.C., Abbot, B.J., Shoemaker, R.H., Litterst, C.L., Hubbard, W.C., Brennan, R.H., Fine, D.L., Eggleston, J.C., Mayo, J.B. and Boyd, M.R. Intrabronchial implantation: a method for the orthotopic propagation of human lung tumors in athymic nude mice. *Chest* 915: 5S-8S, 1987.
2. Shoemaker, R.H., McLemore, T.L., Abbot, B.J., Fine, D.L., Gorelik, E., Mayo, J.G., Fodstad, O. and Boyd, M.R. Human tumor xenograft models for use with an in vitro-based, disease-oriented antitumor drug screening program. In: *Human Tumor Xenografts in Anticancer Drug Development*. European School of Oncology Monograph, in press, 1987.
3. Boyd, M., Shoemaker, R.H., McLemore, T.L., Mayo, J.G., Johnston, M.R., Gazdar, A.F., Minna, J.D. and Chabner, B.A.: Part 7 - Prospects for the Future; Chapter 51 Drug Development. In: Roth, J.A., Ruckleschel, J.C. and Weisenburger, T.H. (eds.), *Thoracic Oncology*, in press, 1987.
4. See also Z01 CM-07162-04 LETM for additional publications.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CM-07178-02 LETM

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell Biology and Developmental Therapeutics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. B. McMahon	Cancer Expert	LETM, NCI
Others: M. R. Boyd	Associate Director	DTP, DCT, NCI

## COOPERATING UNITS (if any)

LEC, NCI (Dr. Thorgeirsson); LEPT, NCI (Dr. Cooney); LC, NCI (Dr. Roberts)

## LAB/BRANCH

Laboratory of Experimental Therapeutics and Metabolism

## SECTION

Pathology and Ultrastructural Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

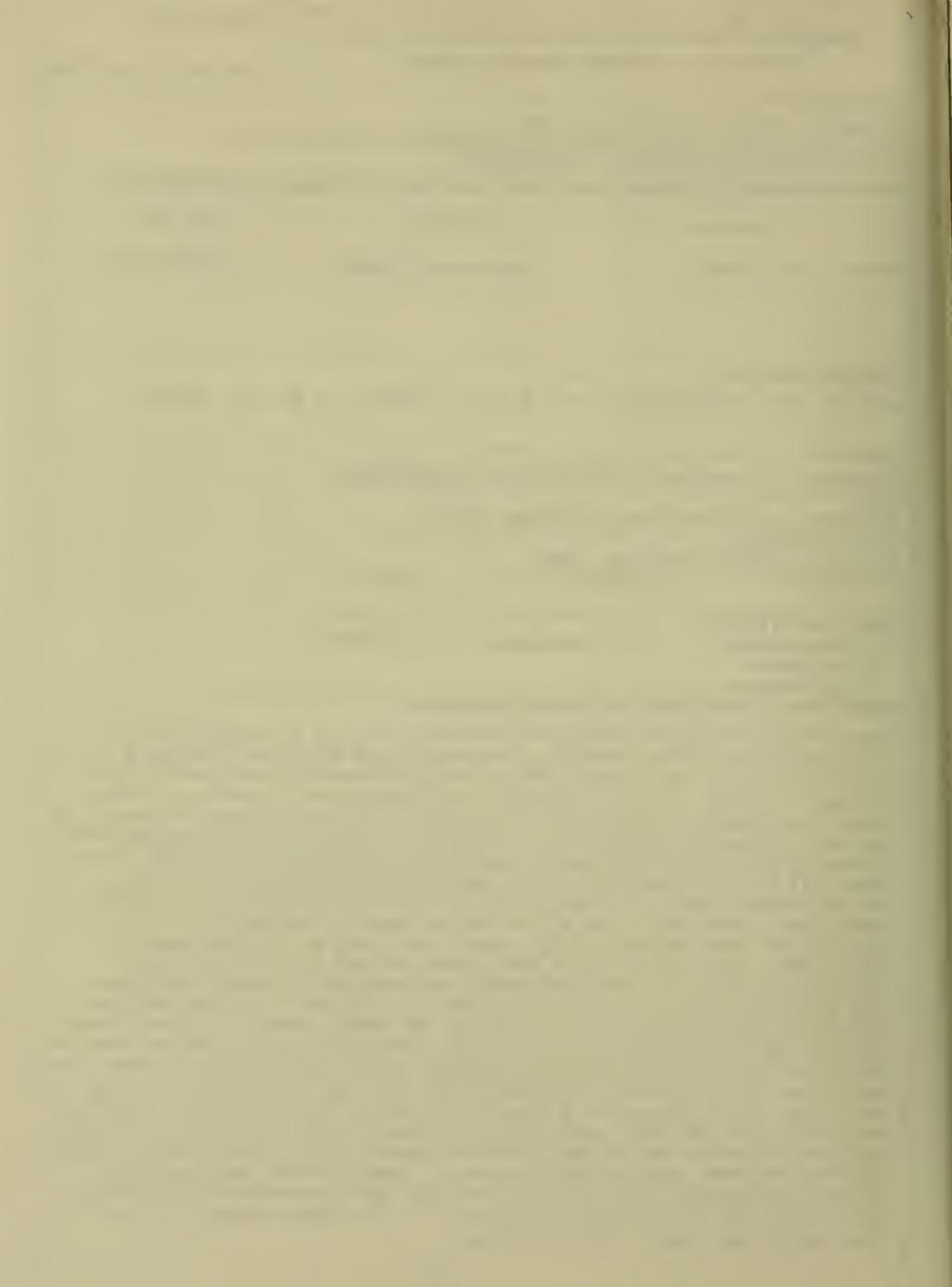
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<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Understanding of the cell biology of cancer may allow us to unravel the carcinogenic process to a point where we can rationally and effectively intervene. This may also facilitate the rational design and development of novel cancer chemotherapeutic agents. We have undertaken a multidirectional approach to carcinogenesis and chemotherapy which involved studies on the cell biology of normal and malignant tissues from both humans and rodents. Methodologies were established to develop and characterize numerous cell lines from normal human kidney tissues. These cell lines include both cortical epithelial cell cultures as well as defined proximal tubular cell populations. The availability of well defined normal human kidney cell lines will allow for detailed pharmacologic and toxicologic studies under defined *in vitro* conditions. Studies directed towards understanding the cell biology of lung cancer have had to rely on the use of human lung cancer cell lines since normal proliferating cultures of peripheral lung epithelium were not available for comparative studies. We have developed new culture methodologies which allowed for the establishment of differentiated alveolar type II cells from rat lung. These developments will allow for detailed studies on the effects of toxins, carcinogens and anticancer drugs on normal lung cells under *in vitro* conditions where proliferation and differentiation can be controlled. Liver cancer cell biology studies which involved chemical transformation of normal rat liver epithelial cells demonstrated that there was a clonal relationship between the two most prominent types of liver cancer, namely hepatocellular carcinomas and cholangiocarcinomas. These findings have now been further substantiated utilizing liver cell-type specific monoclonal antibodies. These studies may lead to the elucidation of the cell types responsible for liver cancer and to more effective therapy.



PROJECT DESCRIPTIONOBJECTIVES

Studies of normal and neoplastic cell biology have shown that carcinogenesis and cancer chemotherapy are closely related from a cellular standpoint. Both of these complex processes are cell type specific and often represent rare cellular events. A thorough understanding of the biology of normal cell types and their alterations during cellular transformation may, therefore, allow for rational approaches to both cancer prevention as well as effective cancer chemotherapy. Extensive studies in rodents have shown that the histogenesis of cancer is a sequential multistep process which results in tumors comprised of a mixture of functionally and morphologically different cell types. The same tumor heterogeneity is often seen in human cancer and may partially explain the lack of sensitivity of most tumor solid tumors to conventional chemotherapy. Pluripotential differentiation of one cell type into another is another property that is common to both the carcinogenesis process and to cells responding to chemotherapy. In order to effectively design therapy for these heterogeneous tumors, the cell biology of the individual cell types and their genotypic and phenotypic interrelationships must be known in detail. The availability and characterization of defined cell populations isolated from normal and neoplastic rodent and human tissues of specific cell types may provide a means to this end.

METHODS EMPLOYED

Human kidney epithelial cell cultures were established as follows: non-diseased cortex tissue was gently cut into small ( $1 \text{ mm}^3$ ) pieces and immersed in RPMI 1640 medium containing 10% fetal bovine serum, 50 ug/ml gentamycin sulfate, and 1 mg/ml type II collagenase. The suspension was stirred mechanically for 90 minutes at 37°C after which time the culture medium was transferred to centrifuge tubes. The larger undigested pieces were allowed to settle out and the supernatant recovered and centrifuged. The cell pellet was resuspended in media and passed over a graded series of screens. The resultant single cell suspension was plated in T25  $\text{cm}^2$  flasks at a density of  $1 \times 10^5$  cells/flask. The cells were incubated at 37°C in humidified cabinets in an atmosphere of 5%  $\text{CO}_2$  in air. Proliferating cultures were characterized biochemically and histochemically by established methods and were subcultured by standard tissue culture techniques. For the isolation and propagation of human kidney epithelial cells of proximal tubule origin the methods of Detrisac et. al. (Kidney International 25: 383-390, 1984) were employed. The proximal tubule cells were identified by employment of enzyme histochemistry, immunohistochemistry, and ultrastructural examination. Proliferation and cytotoxicity were monitored by colony forming and soft agar assay systems. Analysis of DNA content, cell size and specific fluorescence was done by flow cytometry in collaboration with Dr. Cunningham, Bureau of Biologics, FDA. Ultrastructural analysis under various experimental conditions was done with an analytical electron microscope using the transmission mode at 80 KV. Rat alveolar type II cells were isolated and immunologically identified by methods that we have previously described. Modifications to the culture conditions were established which promoted cellular proliferation and inhibited terminal proliferation. Normal rat liver epithelial cells were established from neonatal rat livers by the methods of Herring et. al. (In Vitro 19: 576, 1983). These cells were transformed by continuous exposure to a noncytotoxic



concentration of aflatoxin B<sub>1</sub> for 10 weeks. They were designated AFB cells when the cell line became tumorigenic in syngeneic rats. This cell line was cloned by limited dilution and 7 cell strains that were produced were injected into syngeneic rats. The morphology of the resultant tumors were analyzed by light and electron microscopy. Frozen sections were stained histochemically for gamma glutamyl transpeptidase and immunochemically for liver cell type specific monoclonal antibodies. Cellular proliferation was assayed by several methods including DNA synthesis, cell number and colony formation. Hepatic proliferation inhibitor (HPI) was isolated by a method previously described. TGF-B was provided by Drs. Roberts and Sporn, NCI.

#### MAJOR FINDINGS

- Normal human kidney epithelial cell cultures were successfully established from fresh samples of renal cortex from a number of individual. The collagenase dissociated cells were heterogenous in morphology and possessed a number of heterogenously expressed kidney-specific enzymes. The epithelial cells proliferate rapidly in culture and have been subcultured numerous times, cryopreserved and have not shown signs of sequence.
- Serum-free hormonally defined growth medium was utilized to establish cell cultures of human kidney epithelial cells of proximal tubule origin from a number of individuals. Culture modifications yielded cultures able to be repeatedly subcultured. The cultures were morphologically and biochemically homogenous and were capable of hemicyst formation, a marker for tubular epithelium.
- Culture conditions were successfully developed for the repeated establishment of proliferating rat alveolar type II cell cultures. Under the appropriate conditions the isolated type II epithelial cells are capable of log phase cell division, and subculture without loss of morphological differentiation. The identity of the proliferating cells on type II cells was established using a variety of techniques. These included flow cytometry, microcinematography, light and electron microscopy, analysis of phagocytosis and cytoskeletal components. Furthermore, the culture conditions which favored the rapid proliferation of the isolated type II cells inhibited their terminal differentiation which normally occurred in vitro. Thus, this newly developed system allowed for the control of the proliferation and differentiation of type II cells which mimiced their behavior in vivo.
- TGF-B and HPI were found to be different, but perhaps realted peptides. These findings established HPI as a unique inhibitor of cellular proliferation with biological properties of possible clinical implication.
- Morphological, histochemical and immunohistochemical staining with liver cell-type specific monoclonal antibodies of transformed non-parenchymal rat liver epithelial cells revealed a clonal relationship between cholangiocarcinomas and hepatocellular carcinomas. Furthermore, findings supported the hypothesis that there are multiple cellular lineages leading to liver cancer, some of which may preclude hepatocytes and include pluripotential hepatic stem cells.



- HPI has been shown to inhibit the soft agar-colony formation of normal fibroblasts induced by TGF-B. HPI exerted this effect at pg/ml concentrations and worked independently of the TGF-B or EGF receptor. These findings have broad implications since TGF-B has been shown to exert a wide variety of biological effects in vivo and HPI is the only known peptide to inhibit some of its effects.
- TGF-B was shown to inhibit the proliferation of non-malignant rat liver epithelial cells in culture. The mechanism of this inhibition has now been revealed. TGF-B cause a type of terminal differentiation in this cell type which was characterized by the induction of binucleation, polyploidization, and serum albumin expression as revealed by both immunohistochemical staining and in situ hybridization techniques.

#### SIGNIFICANCE

Cancer in man presents vast problems to the scientist both from a clinical standpoint and from basic cell biology. Therapeutic responses are difficult to predict in part because most human tumors are heterogeneous and often contain malignant cells of different functional and morphological types. The cellular mechanisms responsible for the generation of heterogeneity and the interconversions of cell types seen in malignancy are poorly understood. Detailed studies on the cell biology of normal and neoplastic cells under controlled in vitro conditions may aid in our understanding of these complex processes and help design more appropriate therapies. Moreover, well differentiated cell lines from both normal and neoplastic tissues represent a valuable tool to study cell-type specific biology, biochemistry and pharmacology of identified cell types.

#### PUBLICATIONS

None



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CM 06308-16 BRB

PERIOD COVERED

October 1, 1986 Through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biometric Research Branch

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Sylvain Durrleman, Visiting Researcher

COOPERATING UNITS (if any) Developmental Therapeutics Program, DCT, NCI; Radiation Research Program, DCT, NCI; Biological Response Modifiers Program, DCT, NCI; Clinical Oncology Program, DCT, NCI; Environmental Epidemiology Branch, DCE, NCI.

LAB/BRANCH

Biometric Research Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5

PROFESSIONAL:

4

OTHER:

1.0

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(a) Human subjects       (b) Human tissues       (c) Neither  
 (a1) Minors  
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Biometric Research Branch (BRB) is the statistical component for scientific planning and monitoring of the national and international research program of the Division of Cancer Treatment. The branch provides statistical leadership for all extramural activities of the division. The branch is also responsible for statistical consultation and collaboration with the intramural activities of the Biological Response Modifier Program, Developmental Therapeutics Program, and Radiation Research Program and performs collaborative research with components of the Clinical Oncology Program.

The Biometric Research Branch performs statistical planning and evaluation of all Division of Cancer Treatment supported therapeutic clinical trials. The branch performs scientific monitoring for the statistical aspects of the conduct and analysis of trials performed via cooperative agreement or contract. Primary statistical direction is provided by the branch for the conduct of selected national and international studies of therapeutic interventions, prognostic factors, pre-clinical screening and diagnostic imaging. The branch performs evaluations of therapeutic interventions based upon syntheses of results from multiple studies.

The Biometric Research Branch conducts research on experimental designs, biometric methods and biomathematical approaches for the development and efficient evaluation of improved cancer treatments.











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